



Kreisler 1092-KGB  
001394us/JH/be

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS : GERD HOBOM ET AL.  
SERIAL NO. : 09/909,238  
FILED : July 19, 2001  
FOR : RECOMBINANT INFLUENZA VIRUSES WITH BICISTRONIC  
vRNAs CODING FOR TWO GENES IN TANDEM  
ARRANGEMENT  
ART UNIT : 1614  
EXAMINER : TO BE ASSIGNED

---

February 4, 2002

Hon. Commissioner of Patents  
Washington, D.C. 20231

TRANSMITTAL OF PRIORITY DOCUMENT

SIR:

Appended hereto is a certified copy of Priority Document 00115626.4 filed July 20, 2000.

Applicants request that this document be made of record in the above identified application.

CONDITIONAL PETITION FOR EXTENSION OF TIME

If any extension of time for this response is required, Applicant requests that this be considered a petition therefor. Please charge the required petition fee to Deposit Account No. 14-1263.

**THIS PAGE BLANK (USPTO)**

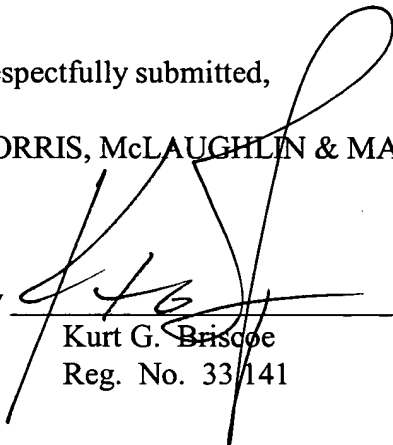
**ADDITIONAL FEE**

Please charge any insufficiency of fees, or credit any excess, to Deposit Account No. 14-1263.

Respectfully submitted,

NORRIS, McLAUGHLIN & MARCUS, P.A.

By

  
Kurt G. Briscoe  
Reg. No. 33/141

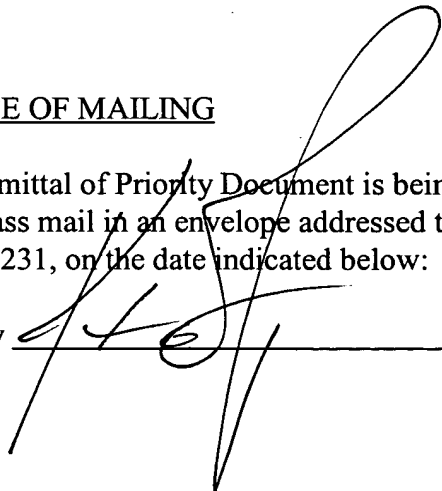
KGB/ja  
220 East 42<sup>nd</sup> Street  
30<sup>th</sup> Floor  
New York, New York 10017  
Tel.: (212) 808-0700  
Fax: (212) 808-0844

**CERTIFICATE OF MAILING**

I hereby certify that the foregoing Transmittal of Priority Document is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Hon. Commissioner of Patents, Washington, D.C. 20231, on the date indicated below:

Date: February 4, 2002

By



**THIS PAGE BLANK (USPTO)**



**Europäisches  
Patentamt**

**European  
Patent Office**

**Office européen  
des brevets**



**Bescheinigung**

**Certificate**

**Attestation**

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

**Patentanmeldung Nr.    Patent application No.    Demande de brevet n°**

00115626.4

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**I.L.C. HATTEN-HECKMAN**

DEN HAAG, DEN  
THE HAGUE,    09/08/01  
LA HAYE, LE

**THIS PAGE BLANK (USPTO)**



Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets

**Blatt 2 der Bescheinigung  
Sheet 2 of the certificate  
Page 2 de l'attestation**

Anmeldung Nr.:  
Application no.: 00115626.4  
Demande n°:

Anmeldetag:  
Date of filing: 20/07/00 ✓  
Date de dépôt:

Anmelder:  
Applicant(s):  
Demandeur(s):  
ARTEMIS Pharmaceuticals GmbH  
51063 Köln  
GERMANY

Bezeichnung der Erfindung:  
Title of the invention:  
Titre de l'invention:

Recombinant influenza viruses with bicistronic vRNAs coding for two genes in tandem arrangement

In Anspruch genomene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:  
State:  
Pays:

Tag:  
Date:  
Date:

Aktenzeichen:  
File no.  
Numéro de dépôt:

Internationale Patentklassifikation:  
International Patent classification:  
Classification internationale des brevets:

C12N15/86, A61K48/00, C12N7/04, A61K39/145

Am Anmeldetag benannte Vertragsstaaten:  
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR  
Etats contractants désignés lors du dépôt:

Bemerkungen:  
Remarks:  
Remarques:

**THIS PAGE BLANK (USPTO)**



20. Juli 2000

000746ep/JH/ml

1

## **Recombinant Influenza Viruses with Bicistronic vRNAs Coding for Two Genes in Tandem Arrangement**

5

### **Field of the Invention**

The invention relates to recombinant influenza viruses for high-yield expression of incorporated foreign gene(s), which are genetically stable in the absence of any helper virus and which comprise at least one viral RNA segment being a tandem bicistronic RNA molecule coding for two genes in tandem, in said tandem bicistronic RNA molecule one of the standard viral genes being in covalent junction with a foreign, recombinant gene and having an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region. In particular the above tandem bicistronic RNA molecule contains one of the standard viral genes in distal mRNA position behind a foreign, recombinant gene in proximal position, or vice versa, both in antisense orientation with regard to the viral RNA within the virus. For simultaneous expression of both genes the proximal reading frame is flanked by splice donor and acceptor signals which have the quality to allow a partial yield of spliced mRNA only, i.e., resulting in the presence of both, spliced and unspliced mRNA simultaneously.

The invention further provides a method for obtaining attenuated viruses which resist reassortment dependent progeny production in case of chance superinfections by wild-type influenza viruses; a method for the production of said recombinant influenza viruses; pharmaceutical compositions comprising said recombinant influenza viruses; and the use of said recombinant influenza viruses for preparing medicaments for vaccination purposes.

## Technical Background

Redesigning influenza virus into a vector system for expression of foreign genes similar to what has been achieved in several other thoroughly studied viruses such as adenovirus, retrovirus, Semliki Forest virus or Rabies virus has the advantage of an industrially well established mode of cheap propagation for influenza in fertilized chicken eggs leading to rather high titers (above  $10^{10}$ /ml). On the other hand none of the constituent vRNA segments may be deleted from the influenza genome according to our present knowledge, and give room for large-size foreign insertions.

Only small fragments of foreign polypeptide chains such as B cell epitopes (10 to 15 amino acids) may be inserted into selected positions within two of the viral proteins, i.e. in exchange for one of the variable antigenic regions located at the surface of hemagglutinin (Muster et al., Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus, J. Virol. 69 (11), 6678-6686 (1995)), or into the stalk sequence of viral neuraminidase (Garcia-Sastre and Palese, The cytoplasmic tail of the neuraminidase protein of influenza A virus does not play an important role in the packaging of this protein into viral envelopes, Virus Res. 37, 37-47 (1995)), and be stably maintained as functional fusion proteins. Constructs of this kind turned out to be useful for experimental vaccination in a few cases studied, but only rather few clearly defined epitope sequences (of ten to twelve amino acids each) are known today, and some of them might also be misfolded within such restricted fusion protein positions, or in other cases interfere with formation of the correct tertiary structure and function of their host polypeptide chains.

Incorporation of a full-size foreign protein into influenza virus via reverse genetics, encoded by an independent ninth vRNA molecule in addition to its regular set of eight standard vRNA segments is without special provisions only transiently possible (Luytjes et al., Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59, 1107-1113 (1989); Enami et al., An influenza virus containing nine

different RNA segments, Virology 185, 291-298 (1991)). In the absence of a continuous selective pressure any additional recombinant vRNA segment cannot be stably maintained as long as the wildtype promoter sequence is used on that ninth vRNA segment, and it will inadvertently be lost after  
5 few steps of viral propagation.

Using a different system of influenza reverse genetics developed in our laboratory (Zobel et al., RNA polymerase I catalysed transcription of insert viral cDNA, Nucleic Acids Res. 21, 3607-3614 (1993); Neumann et al., RNA polymerase I-mediated expression of influenza viral RNA molecules,  
10 Virology 202, 477-479 (1994)), which was built around *in vivo* synthesis of recombinant vRNA molecules by cellular RNA polymerase I transcription of the respective template cDNA constructs, modified terminal viral RNA sequences (hereinafter "promoter-up mutations" or promoter-up variants") have been designed by nucleotide substitutions (Neumann and  
15 Hobom, Mutational analysis of influenza virus promoter elements *in vivo*, J. Gen. Virol. 76, 1709-1717 (1995); WO 96/10641). The above promoter-up variants carry up to five nucleotide substitutions (in promoter-up variant 1920; see Flick and Hobom, J. Gen. Virol. 80, 2565-2572 (1999)). When these promoter-up variants are attached to a  
20 recombinant ninth vRNA segment its increased transcription and amplification rate will not only compensate for the losses suffered spontaneously, but even cause accumulation of the foreign vRNA segment during simple viral passaging, in the absence of any selection.

25 However, due to its over-replication relative to all of the regular influenza vRNA segments (which of course are connected to wild-type promoter sequences) after catching up with the others the foreign segment will become over-abundant. This increasingly will result in viral particles that have incorporated several copies of recombinant vRNA, but no longer have  
30 a full set of all eight standard segments incorporated among an average of about 12-15 vRNA molecules present within a virion. Such particles are defective and will not result in plaque formation, hence after an initial

increase of recombinant viral particles during the first steps of propagation a dramatic decrease is observed, usually at the third or fourth step of viral passaging, depending on the size of the recombinant vRNA and the level of the promoter-up mutation attached.

5

A balanced situation with regard to the insert length and the level of promoter activity can be achieved, and has been propagated in a particular case over 11 passages, with essentially stable levels of recombinant viruses among a majority of helper viruses (around 80%) during these steps. If a full-level promoter-up mutation is used (1104 or the variant 1920, see below) a balanced-level propagation is reached in conjunction with a recombinant vRNA size of 4000 nucleotides (Maysa Azzeh, Ph.D. Thesis, Univ. Giessen (2000)).

10

In all of these preparations, both in transiently achieved increased yields (up to 40% of recombinants after three or four steps of viral passage), and in a balanced propagation of recombinant influenza viruses (10 - 20%) the respective viral progeny inadvertently constitute mixtures with a majority of non-recombinant helper viruses. These result both from a statistical mode of packaging vRNA molecules into a virion (the ninth segment may not be co-packaged), and from the fraction of cells solely infected by helper virus.

15

20

To solve the problems of fractional yields and of instability during viral propagation of recombinant influenza, it was suggested to use a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which comprises at least one viral RNA segment being an ambisense RNA molecule (designated "ambisense RNA segment") and containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement (PCT/EP00/01903). The ambisense RNA segment preferably should contain the promoter-up mutations. The PCT/EP00/01903 moreover discloses a method of constructing specific

25

30

influenza carrier (helper) strains carrying one or more ribozyme target sites (of type one) in vRNA flanking positions comprising

- (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, carrying two different 3' promoter sequences in tandem (an external promoter-up variant and an internal wild-type promoter), which are separated by a second type of ribozyme target sequence, and which carry the said internal ribozyme target sites of type one;
- (b) followed by infection of an influenza wildtype strain;
- (c) thereafter amplification through simple steps of viral propagation; and
- (d) finally isolation through removal of their external 3' promoter sequence by ribozyme cleavage through infection of cells expressing ribozyme type 2, followed by plaque purification.

The resulting special helper virus strains carrying a vRNA segment with external ribozyme target sites of type 1 in exchange for the equivalent regular vRNA molecule are then used for the rescue of ambisense RNA molecules. These are exclusively maintained in the recombinant viruses after passage of viral propagation through ribozyme (type 1) containing host cells, which will destroy the sensitive vRNA molecules of the specially prepared helper viruses.

However, the above ambisense constructs are susceptible to (intra-nuclear) mRNA double-strand formation, which will partially reduce the expression rates of both the ambisense genes, in particular the gene driven by the (weaker) cRNA promoter. The fluctuating extent of this effect made it difficult to bring the expression rate of the influenza gene within the ambisense segment into balance with other influenza genes. This was the problem to be solved with the present invention.

### Summary of the Invention

Starting out from two observations in this laboratory which are discussed above and which concern two hitherto unsuspected properties of influenza

viral RNA polymerase in its interaction with terminally adapted influenza-specific RNA molecules, stable recombinant influenza viruses were found, which solve the above problems.

- 5 The recombinant viruses of the present invention can be used for cheap propagation in fertilized eggs, either for production of those recombinant viruses themselves or for production of foreign proteins or glycoproteins encoded by them, and hence find application in (glyco)protein production or in providing vector systems for somatic gene therapy or in being used  
10 as vaccination agents.

Thus, the present invention provides

- (1) a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper  
15 virus and which comprises at least one viral RNA segment being a bicistronic RNA molecule coding for two genes in tandem arrangement (hereinafter "tandem bicistronic RNA segment" or "tandem RNA segment"), in said tandem RNA segment one of the standard viral genes being in covalent junction with a foreign, recombinant gene and said  
20 tandem RNA segment having an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region;
- (2) a preferred embodiment of the recombinant influenza virus defined in (1) above, in which the terminal viral RNA sequences of said at least one tandem RNA segment, which are active as the promoter signal, have been  
25 modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence;
- (3) a method for the production of recombinant influenza viruses as defined in (1) and (2) above comprising  
30 (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in antisense, or in sense tandem design,

(b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in the corresponding viral RNA segment, i.e., coding for the same viral gene as present in the tandem segment distal position, and

- 5 (c) thereafter selective vRNA inactivation through ribozyme cleavage;
- (4) a pharmaceutical composition comprising a recombinant influenza virus as defined in (1) and (2) above;
- (5) the use of a recombinant influenza virus as defined in (1) and (2) above for preparing a medicament for vaccination purposes;
- 10 (6) the use of a recombinant influenza virus as defined in (1) and (2) above for preparing agents for somatic gene therapy;
- (7) the use of a recombinant influenza virus as defined in (1) and (2) above for preparing agents for transfer and expression of foreign genes into cells (abortively) infected by such viruses;
- 15 (8) the use of a recombinant influenza virus as defined in (1) and (2) above for preparing agents for transfer and expression of RNA molecules into cells infected by such viruses;
- (9) a method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus as defined in (1) and (2)
- 20 above as expression vector;
- (10) a method for preventing and/or treating influenza which comprises administering a recombinant influenza virus as defined in (1) and (2) above to the mammal to be treated, i.e., a vaccination method utilizing said recombinant virus;
- 25 (11) a method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus as defined in (1) and (2) above;
- (12) a method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method
- 30 comprises infecting the cells with a recombinant influenza virus as defined in (1) and (2) above;

(13) use of a recombinant influenza virus as defined in (1) and (2) above for preparing agents for autologous immunotherapy;

(14) a method for an immunotherapy which comprises *ex vivo* infection of immune cells with a recombinant influenza virus as defined in (1) and (2)

5 above, and introduction of the transduced cells into the patient; and

(15) a method for the induction of antibodies which comprises utilizing a recombinant influenza virus as defined in (1) and (2) above as an immunogen.

10 The invention is described in more detail below.

### Brief Description of the Figures

Fig. 1 shows the basepair substitution analysis according to the vRNA

15 'corkscrew' structure:

(A) 'Corkscrew' conformation of the vRNA promoter drawn against a schematic indication of interacting tripartite viral polymerase. Paired positions exchanged in individual experiments are indicated by numbers, nucleotides  $\bar{3}$  or  $\bar{8}$  are counted from the 3' end. pHL2024 containing  
20 promoter-up mutation '1104' is used as the reference construct (=100%) in all of the CAT assays, while pHL2428 represents the wild-type promoter structure.

(B) CAT analysis of a series of substitution variants in positions 3 and 8 from the 5' end as indicated above the lanes; 50  $\mu$ l of cell lysate obtained  
25 from  $10^6$  MDCK cells infected in the first viral passage with recombinant viral progeny.

(C) pHL2024 and pHL1920 comparative CAT analysis, in 100 fold dilution relative to (B), i.e., obtained from 0.5  $\mu$ l of cell lysate in 3 h reaction time.

30 Fig. 2: Vector plasmid pHL1920, the exact sequence of the 3888 bps circular DNA is shown in SEQ ID NO: 20



Fig. 3 shows the genetic structure and the RNA transcription products of influenza model tandem expression constructs. Heavy lines for the plasmid cDNA constructs refer to double-stranded DNA, while single-stranded RNA molecules are represented by thin lines, and their 5' to 3' 5 directionality is marked by arrows. Standard modifications at their 5' and 3' ends are indicated by a dot (5' cap structure) and A<sub>n</sub> (3' polyadenylation), both are absent in the primary anti-sense transcription product, the viral RNA (vRNA), which is transcribed by cellular RNA polymerase I (RPOI). Full-length mRNA, is synthesized by influenza viral polymerase (virPo), and a partial splice reaction results in a functional yield of shorter mRNA<sub>2</sub> molecules. While both of the reporter genes are indicated on the DNA level, together with the positions of splice donor (D) and acceptor (A) signal sequences as well as the promotor (p<sub>I</sub>) and terminator (t<sub>I</sub>) elements for RNA polymerase I start and stop, on the RNA level only those genes and splice signals are marked that are actually translated into protein or actively involved in splicing. The chloramphenicol-acetyltransferase gene (CAT) has been inserted in proximal position in pHL3196 and pHL3235, and in distal position in pHL3224 and pHL3236 (see Figs. 5 to 8), while green fluorescent protein (GFP) in each case is located in alternate location. All vRNA molecules - and hence, also the cDNA constructs - carry sequence variations at their 3' ends, which together constitute the 1104 promoter-up mutations: G3A, U5C, C8U (nucleotide positions counted from the 3' vRNA end). pHL3235 and pHL3236 vRNAs are extended in size by about 1000 nucleotides of untranslated sequence relative to pHL3196 and pHL3226: 2600 instead of 1600 nucleotides in lengths. For full-size representation of circular plasmid DNAs see Figs. 5-8, for CAT expression data of all infected by recombinant influenza viruses carrying the respective viral RNAs see Fig 4.

30

Fig. 4 shows the CAT assay results for the group of tandem vRNA plasmid constructs as described in the Example. In particular, the ratio between

chloramphenicol (bottom line) and acetylchloramphenicol (upper three lines) in a flash-CAT assay, after the 2<sup>nd</sup> (A) and 4<sup>th</sup> (B) passage of recombinant viruses carrying the reportergene CAT, can be determined from said figure. The following constructs were utilized:

5 pHL1844 (control): monocistronic CAT-construct downstream of p<sub>promoter</sub>-variant 1104.

pHL3196: tandem construct, p-CAT-GFP resulting in a vRNA having a total length of 1530 nucleotides (not "extended"), see also Fig. 5.

10 pHL3235: tandem construct, p-CAT-GFP resulting in a vRNA having a total length of 2550 nucleotides ("extended"), see also Fig. 7.

pHL3224: tandem construct, p-CAT-GFP resulting in a vRNA having a total length of 1700 nucleotides (not "extended"), see also Fig. 6.

pHL3236: tandem construct, p-CAT-GFP resulting in a vRNA having a total length of 2720 nucleotides ("extended"), see also Fig. 8.

15 pHL2899: ambisense construct, p<sub>v</sub>-CAT→ ←GFP-p<sub>c</sub> resulting in an RNA having a total length of 1500 nucleotides.

pHL2960: ambisense construct, p<sub>v</sub>-CAT→ ←GFP-p<sub>c</sub> resulting in an RNA having a total length of 1500 nucleotides.

The five constructs on the left side were transfected into the cell DNA  
20 without the use of "booster" plasmides, the four constructs on the right side were, however, transfected with the "booster" plasmides, which gives a jump-start of the constructs due to recombinant vRNA amplification prior to helper virus injection, equivalent to an advantage of about two passages. The "booster" plasmides comprise expression constructs for the  
25 nucleoprotein as well as the three subunits of influenza viral polymerase, each downstream of an RNA polymerase II promoter and in an mRNA forming cassette.

While the ambisense construct having the CAT-reporter gene in the weaker position, i.e. behind the cRNA promoter (pHL2899), is only  
30 expressed moderately, this is not the case in the respective tandem construct having the CAT-reporter gene in the weaker position, viz.

pHL3224 or pHL3236. Further, the "extension" of the vRNA by 1020 non-translated nucleotides (at the 3' end) is tolerated without significant decrease of expression (see pHL3235 versus pHL3196).

- 5 Fig. 5: Vector plasmid pHL3196, the exact sequence of the 4500 bps circular DNA is shown in SEQ ID NO:21.

Fig. 6: Vector plasmid pHL3224, the exact sequence of the 4721 bps circular DNA is shown in SEQ ID NO:22.

10

Fig. 7: Vector plasmid pHL3235, the exact sequence of the 5517 bps circular DNA is shown in SEQ ID NO:23.

- 15 Fig. 8: Vector plasmid pHL3236, the exact sequence of the 5699 bps circular DNA is shown in SEQ ID NO:24.

### **Detailed Description of the Invention**

20 According to the present invention "influenza virus" embraces influenza A virus, influenza B virus and influenza C virus, with influenza A virus being preferred.

"Bicistronic" according to the present invention refers to a viral RNA segment, vRNA, cRNA or mRNA that includes two independent genes in covalent junction; in a preferred version one of these genes is of viral origin, while the other one codes for a foreign, recombinant gene product.

25 "Proximal" and "proximal position" according to the present invention refers to the 5' portion of one of the genes in the bicistronic viral mRNA, i.e., ahead (upstream) of the second gene in "distal position".

30

A "mammal" according to the present invention includes humans and animals. "Organism" embraces prokaryotic and eukaryotic systems as well

as multicellular systems such as vertebrates (including mammals) and invertebrates.

"Infected cells" and "infecting cells" according to the present invention  
5 also include "abortively infected cells" and "abortively infecting cells", -  
respectively.

In a preferred influenza virus according to embodiment (1) at least one of  
the regular viral RNA segments is replaced by a tandem RNA segment  
10 which contains one of the standard viral genes in distal position, and a  
foreign, recombinant gene in proximal position, both in anti-sense  
orientation, or vice-versa. It is moreover preferred that in the tandem RNA  
molecule said foreign recombinant gene is covalently bound to one of the  
viral genes while the original vRNA segment coding for the same gene is  
15 deleted from the recombinant virus by specific ribozyme cleavage.

The foreign gene(s) in tandem covalent junction with the viral gene(s)  
preferably code for proteins and/or glycoproteins which are secreted from  
cells infected with the recombinant virus, such as lymphokines, or code for  
20 glycoproteins that are incorporated into the virion as well as the plasma  
membrane of the infected cell. In another preferred embodiment the  
foreign gene(s) in tandem covalent junction with the viral gene(s) code for  
proteins or artificial polypeptides designed to support an efficient  
presentation of inherent epitopes at the surface of infected cells, for  
25 stimulation of B cell and/or T cell response. Such proteins or artificial  
polypeptides constitute for instance a tumor antigen or an artificial  
oligomeric series of T cell epitopes that have been identified within a  
polypeptide chain. Finally, the foreign gene(s) may be suitable for transfer  
and expression of RNA molecules, including antisense RNAs and  
30 ribozymes, into cells. Such recombinant influenza viruses are suitable for  
sequence specific gene silencing, for example by antisense or RNA  
interference mechanisms.

A preferred recombinant virus of the invention is where in the regular viral RNA segments one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged, preferably into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

As set forth in embodiment (2) above, a preferred recombinant virus of the invention is where the terminal viral RNA sequences, which are active as promoter signal, have been modified by nucleotide substitution in up to 5 positions, resulting in improved transcription rates (of both the vRNA promoter and in the cRNA promoter as present in the complementary sequence) as well as enhanced replication and/or expression rates relative to the wild-type sequence. Said modified terminal viral RNA sequences differ from the wild-type sequence in that in said tandem vRNA segment the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides provided that the nucleotides introduced in positions 3 and 8 are forming a base pair (i.e., if the nucleotide position 3 is G, than that in position 8 is C; if the nucleotide in position 3 is C, than that in position 8 is G; etc.).

The 3' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: (5')-CCUGCUUUUGCU-3'

Influenza B: (5')-NN(C/U)GCUUCUGCU-3'

Influenza C: (5')-CCUGCUUCUGCU-3'.

Moreover, the 13 nucleotide conserved influenza 5'-terminal sequence may be modified by replacement of one or two nucleotides occurring in said sequence as positions 3 and 8 by other nucleotides, again provided that the introduced nucleotides are forming a base pair. The 5' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: 5'-AGUAGAAACAAGG

Influenza B: 5'-AGUAG(A/U)AACA(A/G)NN

Influenza C: 5'-AGCAGUAGCAAG(G/A):

Preferred influenza viruses of the invention are those wherein in the 3' conserved region the replacements G3A and C8U have been performed, more preferred are those where also the replacement U5C has been performed (the above mutations are annotated relative to the 3' end; such counting from the 3' end is also indicated by a line on top of the digit, e.g., G  $\bar{3}$ A). Another preferred influenza virus mutant comprises the 3'-terminal nucleotide sequence G3C, U5C and C8G (relative to the 3' end) resulting in the following 3' terminal nucleotide sequence (5')-CCUGGUUCUCCU-3'. Among the influenza viruses defined hereinbefore those having a 3'-terminal nucleotide sequence of (5')-CCUGUUUCUACU-3' are most preferred. In case of an influenza A virus the segment may further have the modifications U3A and A8U in its 5' terminal sequence, in case of influenza C it may have the modifications C3U and G8A in its 5' terminal sequence. The most preferred influenza viruses of the present invention comprise the following general structures:

Influenza A (mutant pHL1104):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUG $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{C}}$  $\overline{\text{U}}$ ACU-3'

Influenza A (mutant pHL1920):

5'-AG $\overline{\text{A}}$ AGAA $\overline{\text{U}}$ CAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUG $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{C}}$  $\overline{\text{U}}$ ACU-3'

Influenza A (mutant pHL1948):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUG $\overline{\text{G}}$  $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{C}}$  $\overline{\text{U}}$ CCU-3'

Influenza B:

5'-AGUAG(A/U)AACA(A/G)NNNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'N'(C/U)GUUUCUACU-3'

Influenza C:

5'-AGUAGUAACAAG(G/A)GU<sub>5-6</sub>..(880-2300 ntds)..CCCCUGUUUCUACU-3'

5

In the above structures the variables are defined as follows:

- (1) Underlined and enlarged letters show the required mutations relative to the wild-type sequence for preparing a promoter mutant with enhanced properties;
- 10 (2) enlarged A in position 10 in the 5'-part of the sequence: unpaired A residue, bulge-forming;
- (3) (A/G) in one position: different isolates or single segments with variable sequence at the respective position, which are functionally interchangeable;
- 15 (4) N and N': positions undefined, but base-paired relative to each other because of complementarity between the 5' and 3' termini, different among the 8 segments, but constant for each segment throughout all viral isolates;
- (5) (880-2300 ntds): the lengths of the viral RNA segments, in case of
- 20 segments with foreign genes increased up to 4,000 nucleotides.

According to embodiments (1) to (3) the invention provides

- a stable recombinant influenza virus containing (up to) seven regular vRNA segments plus one (or more) additional bicistronic segment(s)
- 25 coding for a foreign gene in covalent conjunction with one of the influenza genes, in tandem arrangement, and
- a method for the construction of stable recombinant influenza viruses through tandem arrangement of bicistronic vRNA segments, which is also applicable as a method for attenuation and for prevention of reassortment
- 30 between co-infecting influenza viruses.

Expression of both gene products in these constructions is made possible by way of an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region of such a quality that splicing does occur in part of the mRNA molecules only, i.e., both mRNAs  
5 spliced and unspliced are present in the infected cell. For compensation with regard to the vRNA length the bicistronic segment is connected to a promoter variant of enhanced replication and transcription rates as defined herein before.

- 10 The splice donor and the splice acceptor signals are selected from authentic sequences as present in influenza segments 7 and 8 or other partially effective splice reaction substrates, preferably those of influenza virus WSN segment 7, i.e., 5'-AG<sup>↓</sup>GTACGTTC-3' (donor) and 5'-GCTGAAAAATGATCTTCTTGAAAATTGCAG<sup>↓</sup>GC-3' (acceptor).

15

- In a particular application of embodiments (1) to (3) the tandem bicistronic mRNA codes for one of the viral genes, such as hemagglutinin, in conjunction with all or part of the viral neuraminidase coding sequence, in antisense orientation, while the authentic neuraminidase vRNA segment  
20 is missing in these recombinant viruses. In another variation of these constructs an anti-neuraminidase ribozyme sequence is also provided together with the (partial) neuraminidase antisense sequence, in the proximal position of these bicistronic recombinant segments. Recombinant viruses of this character are propagated in culture media with addition of  
25 exogenous neuraminidase.

- The absence of a functional neuraminidase gene serves as a strong attenuation mechanism resulting in single-step infections of such recombinant viruses only. While a functional neuraminidase gene could be  
30 provided through another (wildtype) influenza virus superinfecting the same cell, expression of that gene is very much reduced through antisense RNA interaction and/or destruction of the corresponding vRNA



through ribozyme cleavage, designed to interfere with production of infectious progeny even from co-infected cells; as a barrier against reassortment in double infected cells.

- 5 Recombinant viral RNAs coding simultaneously for two genes in tandem in a construct in which one of the viral genes is in covalent junction with a foreign coding sequence, are constructed via E. coli plasmid vector DNAs designed for an *in vivo* transcription of minus-strand vRNAs by cellular RNA polymerase I. In these constructs the gene in plus-strand proximal  
10 (upstream) position is surrounded by splice signals of limited activity such that both mRNAs, spliced and unspliced are present in the infected cell. Either the foreign gene or the viral gene may be in that upstream position. In the majority of applications the higher rates of expression will be reserved for the foreign coding sequence, while the lower expression rate  
15 of the viral gene is adapted to be approximately in balance with expression of the other viral genes encoded by the regular viral segments.

To achieve such a balanced rate of expression, the splice signals and the promoter have to be chosen properly (Flick and Hobom, Interaction of  
20 influenza virus, polymerase with viral RNA in the 'corkscrew' conformation, J. Gen. Virol. 80, 2565-2572 (1999)). At an increased overall transcription rate, the resulting mRNAs shall be spliced inefficiently if the viral gene is in the distal (downstream) position. Vice-versa, if the foreign gene is in the distal position, splicing to obtain the foreign mRNA shall be achieved  
25 efficiently. Both designs serve to reach an over-expression of the foreign gene relative to the viral gene, of which the expression shall be in balance with the expression of the other viral genes. Further, the promoter variant attached to the bicistronic segment has the function to compensate for the increased gene length by way of an increased replication rate.

30

The influenza vRNA segments preferably used for construction of bicistronic segments include the neuraminidase (No. 6), hemagglutinin

(No. 4) and NS segment (No. 8). In the NS segment the foreign gene may also substitute for the NS1 gene leaving the viral NS2 gene in its place. These recombinant viruses can, as an example, be made by the following procedure: A recombinant virus population can be selected by repeated  
5 ribozyme-mediated cleavage of helper-virus segments carrying ribozyme cleavage sites that flank the same viral gene in the monocistronic segment as is present in the bicistronic construct (PCT/EP00/01903). By serial viral passaging and relying on the output of reporter genes in equivalently constructed bicistronic segments, a balanced mode of expression can be  
10 achieved in choosing the right set of elements: promoter, splice signals plus a limited variation in segment length. The construct that gives rise to the balanced, stable expression is then used as a basis for a multiple cDNA transfection procedure in a helper-virus free design according to Neumann et al., Proc. Natl. Acad. Sci. USA, Vol 96, 9345-9350 (August 1999). The  
15 resulting recombinant influenza virus, obtained via single plaques in pure helper-free state is subjected to another series of propagation steps to finally evaluate its properties.

In a particular application this design is used for a controlled mode of viral  
20 attenuation. Attenuation of influenza viruses so far has been achieved in cold-sensitive mutants (Edwards et al., J. Infect. Dis. 169, 68-76(1994)), by deletion of the NS1 gene (partial attenuation, Egorov et al., J. Virol. 72, 6437-6441 (August 1998) and Palese et al., Proc. Natl. Acad. Sci USA, 4309-4314 (April 2000)), or through deletion of the neuraminidase gene  
25 (full attenuation, Kawaoka et al., J. Virol. 74, 5206-5212 (June 2000)). The latter approach is adapted here using a novel technique for the attenuation, which for the first time is also able to interfere with (chance) superinfection by wild-type viruses.

30 In this embodiment of the invention a bicistronic cDNA construct is achieved, which instead of a foreign gene is coding either for part of or for the entire viral neuraminidase gene in antisense orientation, with or

without being surrounded both by splice donor and acceptor elements. In another version of that design a 2 x 50 nucleotide antisense segment complementary to the 5'-terminal neuraminidase sequence has been cloned in flanking positions relative to a ribozyme construct according to the hammerhead design and oriented against a common GUC-triplett within the neuraminidase sequence. In a preferred design this antisense expression construct has been attached to the hemagglutinin vRNA segment, while another gene or reporter gene is encoded in a second bicistronic vRNA, in conjunction with NS2.

10

Propagation of recombinant viruses deleted for the neuraminidase (NA) gene requires an addition of external neuraminidase to the medium. In the absence of neuraminidase, infection by the NA deletion viruses is abortive: no infectious progeny is produced. Upon co-infection (3:3) of recombinant viruses together with wildtype viruses no progeny virus or plaque is observed, which is attributed to antisense-blocked expression or (partial) destruction of the neuraminidase segment originating from the wild-type virus. Therefore, the recombinant viruses described are not only attenuated in single infections, but simultaneously interfere with wildtype virus superinfection, and therefore, no re-assortment between the two viruses will occur.

15  
20

The pharmaceutical composition according to embodiment (4) above and the medicament of embodiment (5) above contain the recombinant influenza virus in a pharmaceutically effective amount. Besides said recombinant influenza virus, the pharmaceutical composition and the medicament may contain further pharmaceutically acceptable carrier substances well-known to a person skilled in the art, such as binders, desintegrants, diluents, buffers, preservatives, etc. The pharmaceutical composition and medicaments are solid or liquid preparations and are suitable to be administered orally, intravenously or subcutaneously.

25  
30

The medicament according to embodiment (5) above is preferably suitable as a medicament against influenza and/or against other infections. The recombinant influenza virus may be present in form of inactivated preparations or may be present in form of live recombinant viruses, preferably as attenuated viruses.

Live recombinant viral vaccines, live but attenuated recombinant viral vaccines or inactivated recombinant viral vaccine can be formulated. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity is destroyed without affecting its immunogenicity. To prepare inactivated vaccines, the recombinant virus may be grown in cell cultures or in embryonated chicken eggs, purified, and inactivated by formaldehyde or  $\beta$ -propiolactone. The resulting vaccine is usually administered intramuscularly.

Inactivated viruses may be formulated with suitable adjuvants to enhance the immunological response. Such adjuvants include, but are not limited to, mineral gels, e.g., aluminum hydroxide, surface-active substances such as pluronic polyols, lysolecithin, peptides, oil emulsions, and potentially useful human adjuvants such as BCG.

Many methods may be used to introduce the vaccine formulations above, for example the oral, intradermal, intramuscular, intraperitoneal, subcutaneous, or intranasal routes. Where a live recombinant virus vaccine is used, it is preferred to introduce the formulation via the natural route of infection for influenza virus.

The medicament according to embodiment (5) above is preferably suitable for prophylactic or therapeutic vaccination, or both, against influenza and other infections. For example, recombinant viruses can be made for use in vaccines against HIV, hepatitis B virus, hepatitis C virus, herpes viruses, papilloma viruses, to name but a few. In one embodiment the

recombinant virus contains the genes for surface proteins of the viruses, in another the genes for non-structural or regulatory genes. The recombinant viruses may be present in form of inactivated preparations or may be present in form of live recombinant viruses, or as live, but attenuated viruses. In an attenuated virus the recombinant virus would go through a single or at most very few propagation cycle(s) and induce a sufficient level of immune response, but would not cause disease. Such viruses lack one of the essential influenza genes or contain mutations to introduce temperature sensitivity.

10 The agents of embodiments (6)-(8) of the invention are applicable in *ex vivo* and *in vivo* application schemes. The RNA molecule to be expressed by means of the agent of the embodiment (8) is of an antisense sequence or double strand sequence (in ambisense bidirectional transcription) relative to a target cellular mRNA molecule. In embodiment (8) the agent is preferably suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

20 The method for the production of proteins or glycoproteins is preferably performed in cell culture cells or in fertilized chicken cells in accordance with standard techniques within the general knowledge of a person skilled in the art. The proteins or glycoproteins to be expressed are those incorporated into the ambisense construct as defined hereinbefore.

25 The methods according to embodiments (9) to (12), (14) and (15) of the invention include the administration of an effective amount to the mammal or the administration of a sufficient infective dose of the recombinant virus to the cell system that is used for *ex vivo* therapy or for *in vitro* investigations, whereby the amount and dose will be determined by a person skilled in the respective arts or knowledgeable of the desired treatments.

The agent of embodiment (14) of the invention is preferably utilized to infect, transfect or transduce patient-derived immune cells. The agent is suitable for treatment of cancer or chronic viral infections. For this purpose, patient derived immune cells, preferably dendritic cells, are ex vivo infected with recombinant influenza viruses expressing, e.g., tumor antigens or viral antigens. The transduced cells are then reintroduced into the patient.

The preferred method for immunotherapy of embodiment (14) of the invention is an autologous immunotherapy, wherein the cells which are ex vivo infected are patient-derived and the transduced cells are reintroduced into the patient. The diseases to be treated by this method include cancer and chronic viral infections. For details regarding such treatment see discussion of embodiment (13) above.

The method for inducing antibodies according to embodiment (15) of the invention is suitable for inducing antibodies to foreign proteins including glycoproteins, following the administration of protein or glycoprotein antigens as part of a recombinant influenza virus in an authentic conformation, whereby the virus is purified by gentle procedures based on hemagglutination, and the gene is expressed at high rates in the infected cells.

As influenza viruses have a wide host range, recombinant influenza viruses can be used to obtain strong immune responses in, and isolate antibodies from, a wide range of animals, including, but not limited to, fowl, pigs, horses, and mice. Further, influenza viruses adapted to the mouse can be used for the infection of mice by several routes including the intranasal route. This results in infection of the pharyngeal mucosal cells and results in an additional type of B cell response (e.g., as recognized in the ratio of IgG to IgA). Mice are of particular utility in the induction of immune responses in transgenic mice that have been

engineered to express human antibodies. As gentle procedures based on hemadsorption are used to purify influenza viruses, antibodies to antigens in native conformation can be isolated from the infected mammals.

The preset invention further illustrated by the following, non-limiting

5 Example:

### Example

Model tandem bicistronic expression constructs using reporter genes CAT and GFP.

- 10 Objective: Measurements of relative expression rates for CAT in proximal and distal position, with live observation of GFP fluorescence in alternate position during propagation of recombinant influenza viruses.

#### a) Construction of bicistronic expression plasmid DNAs:

- Starting out with vector plasmid pHH10 (Hoffmann, Ph.D. Thesis, Univ. Giessen (1997)), i.e. an ampicillin resistant plasmid including in between a human rDNA promoter segment and a murine rDNA terminator segment precisely inserted cDNA sequence elements representing the 5' and 3' vRNA sequence of influenza rRNA segment 5, and finally a central multiple cloning site sequence as obtained from plasmid PBSK, both reporter genes
- 15 have been inserted in a stepwise manner. After that, to the proximal reading frame, i.e. CAT in pHL3196, and GFP in pHL3224, has been added an upstream splice donor sequence element and a downstream splice acceptor element, both inserted as double-strand oligonucleotides, in between particular restriction cleavage sites available in the respective
- 20 positions. The signal sequences used in that pair of plasmids indicated above have been derived from influenza vRNA segment 7, which is known for its partial splice reactions yielding both gene products, M1 and M2, simultaneously. By insertion of a non-transcribed DNA fragment (representing an internal segment of the influenza PB1 coding region) in a
- 25 distal position relative to both reading frames, pHL3196 has been converted into pHL3235, and pHL3224 into pHL3236. For the resulting plasmid constructs see Figs 5-8 and SEQ ID NOs: 21-24.
- 30

b) Transfection of plasmid DNAs and isolation of recombinant influenza viruses:

Semi-confluent 293-T cells, a human renal cacinoma cell line carrying an artificially integrated tumor virus SV40 T-antigen gene, were DNA-transfected using lipofectamine: 5-10  $\mu\text{g}$  of DNA mixed with 30  $\mu\text{l}$  Lipofectamine® (GIBCO/BRL) were added to 370  $\mu\text{l}$  of DMEM medium and were incubated with  $5 \times 10^6$  to  $10^7$  cells, washed and maintained serum-free for 5 to 8 hours, before serum was added for another 12 to 15 hours. Finally influenza helper virus FPV<sub>Bratislava</sub> was used for infection of the DNA-transfected cells. The supernatant containing a mixture of helper viruses and recombinant viruses was collected for further propagation after 8 to 12 hours of infection, while the sedimented cells were used for preparation of a cell lysate, fractions of which were inserted in the CAT assay procedure.

Viral propagation was achieved by infection of MDCK cells (Madin-Darby canine kidney cell line) again in semi-confluent state ( $5 \times 10^6$  to  $10^7$  cells per plate), generally using 1ml of the previous supernatant for infection. Serial propagations were done in the same way, with preparation of cell lysates for CAT assays at the end of each step. Infected cells were also used for observation of GFP fluorescence.

c) CAT assay:

Bacterial chloramphenicol-acetyltransferase (CAT) is accumulated in eukaryotic cells without degradation and can be used for representative gene expression measurements. The substrate used here is fluorescent boron-dipyrromethane-chloramphenicol diflouride (FLASH CAT-KIT®; Stratagene). 50  $\mu\text{l}$  of cell lysate or reduced/diluted samples thereof were used for incubation with 7.5  $\mu\text{l}$  of fluorescent substrate and 10  $\mu\text{l}$  acetyl-CoA (4mM) co-substrate in 19 mM Tris/HCl, pH: 7.5 at 37°C for 3 hours. For extraction of reaction products 1ml of ethylacetate ins added, the



mixture is vortexed, and separated by centrifugation. After solvent evaporation and dissolution again in 20ml ethylacetate, the reaction products are separated on a silica thin-layer chromatography plate using chloroform/methanol 87:13% (vol.) and the results are documented by photography under UV light.

#### d) Results

CAT in proximal or in distal position of this pair of recombinant plasmids is expressed about equally (Fig.4), and the same is true for GFP (not shown). The expression rates are increasing during the initial steps of viral propagation and stay about constant afterwards during further steps of recombinant viral passages, different from expression rates in ambisense bicistronic constructs (pHL2899 and pHL2960) (Fig. 4B). Co-transfection of booster plasmids in the initial 293-T cells increase the yields of recombinant viruses within the progeny population, which are maintained during consecutive steps of propagation. Addition of 1000 nucleotides of untranslated vRNA sequence will not reduce the expression rates substantially (pHL3235 versus pHL3196, and pHL3236 versus pHL3224).

**THIS PAGE BLANK (USPTO)**

20. Juli 2000

26

## SEQUENCE LISTING

&lt;110&gt; ARTEMIS PHARMACEUTICALS GmbH

5 <120> Recombinant Influenza Viruses with Bicistronic vRNAs Coding for  
Two Genes in Tandem Arrangement

&lt;130&gt; C00746ep/JH/ml

10 <140>  
<141>

&lt;160&gt; 24

15 &lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 12

&lt;212&gt; RNA

20 &lt;213&gt; Influenza A virus

&lt;400&gt; 1

ccugcuuuug cu

12

25

&lt;210&gt; 2

&lt;211&gt; 12

&lt;212&gt; RNA

&lt;213&gt; Influenza B virus

30

&lt;400&gt; 2

nnygcuucug cu

12

35

&lt;210&gt; 3

&lt;211&gt; 12

&lt;212&gt; RNA

&lt;213&gt; Influenza C virus

40

&lt;400&gt; 3

ccugcuucug cu

12

45

&lt;210&gt; 4

&lt;211&gt; 12

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence -

50 &lt;220&gt;

<223> Description of Artificial Sequence: Modified  
influenza A 5'-sequence (pHL1104 and pHL1920)

27

<400> 4  
ccuguuucua cu 12

5 <210> 5  
<211> 12  
<212> RNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: Modified  
influenza A 3'-sequence (pHL1948)

<400> 5  
15 ccugguucuc cu 12

<210> 6  
<211> 13  
20 <212> RNA  
<213> Influenza A virus

<220>  
<223> Description of Artificial Sequence: Modified  
25 influenza A 3'-sequence (pHL1948)

<400> 6  
aguagaaca agg 13

30 <210> 7  
<211> 13  
<212> RNA  
<213> Influenza B virus

35 <400> 7  
aguagwaaca rnn 13

40 <210> 8  
<211> 13  
<212> RNA  
<213> Influenza C virus

45 <400> 8  
agcaguagca agr 13

<210> 9  
50 <211> 13  
<212> RNA  
<213> Artificial Sequence

28

&lt;220&gt;

<223> Description of Artificial Sequence: Modified  
influenza A 5'-sequence (pHL1920)

5	<400> 9 agaagaauca agg	13
10	<210> 10 <211> 21 <212> RNA <213> Influenza A virus	
15	<400> 10 aguagaaaca aggnnnuuuu u	21
20	<210> 11 <211> 21 <212> RNA <213> Artificial Sequence	
25	<220> <223> Description of Artificial Sequence: Modified influenza A 5'-sequence (pHL1920)	
30	<400> 11 agaagaauca aggnnnuuuu u	21
35	<210> 12 <211> 21 <212> RNA <213> Influenza B virus	
40	<400> 12 aguagwaaca rnnnnuuuu u	21
45	<210> 13 <211> 19 <212> RNA <213> Artificial Sequence	
50	<220> <223> Description of Artificial Sequence: Modified influenza C 5'-sequence	
50	<400> 13 aguaguaaca agrguuuuu	19

29

<210> 14  
<211> 15  
<212> RNA  
<213> Artificial Sequence  
5  
<220>  
<223> Description of Artificial Sequence: Modified  
influenza A 3'-sequence (pHL1104 and pHL1920)  
10 <400> 14  
nnnccuguuu cuacu 15  
  
<210> 15  
15 <211> 15  
<212> RNA  
<213> Artificial Sequence  
  
<220>  
20 <223> Description of Artificial Sequence: Modified  
influenza A 3'-sequence (pHL1948)  
  
<400> 15  
nnnccugguu cuccu 15  
25  
  
<210> 16  
<211> 15  
<212> RNA  
30 <213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Modified  
influenza B 3' sequence  
35  
<400> 16  
nnnnnyguuu cuacu 15  
  
<210> 17  
40 <211> 14  
<212> RNA  
<213> Artificial Sequence  
  
<220>  
45 <223> Description of Artificial Sequence: Modified  
influenza C 3'-sequence  
  
<400> 17  
50 ccccguuuc uacu 14

30

<210> 18  
 <211> 10  
 <212> DNA  
 <213> Influenza A virus

5  
 <400> 18  
 aggtacgttc 10

10 <210> 19  
 <211> 32  
 <212> DNA  
 <213> Influenza A virus

15 <400> 19  
 gctgaaaaat gatcttcttg aaaattgcag gc 32

20 <210> 20  
 <211> 3888  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 25 <223> Description of Artificial Sequence: pHL1920

<400> 20  
 cccaaaaaaa aaaaaaaaaa aaaaaaaaaag agtccagagt ggccccgcgcg ttccgcgcgcg 60  
 gggggggggg gggggggggg cacttttcgga catctggtcg acctccagca tcgggggaaa 120  
 30 aaaaaaaaaa aaagtttcgc ccggagtact ggtcgacctc cgaagttggg ggggagtaga 180  
 aacagggttag ataatacactc actgagtgac atccacatcg cgagcgcgcg taatacgact 240  
 cactataggg cgaattgggt accgggcccc ccctcgaggt cgacggtatc gataagcttc 300  
 gacgagattt tcaggagcta aggaagctaa aatggagaaa aaaatcactg gatataccac 360  
 cgttgaataa tcccaatggc atcgtaaaga acattttgag gcatttcagt cagttgctca 420  
 35 atgtacctat aaccagaccg ttcagctgga tattacggcc tttttaaaga ccgtaaagaa 480  
 aaataagcac aagttttatc cggcctttat tcacattctt gcccgctga tgaatgctca 540  
 tcgggaattc cgtatggcaa tgaaagacgg tgagctgggtg atatgggata gtgttcaccc 600  
 ttgttacacc gttttccatg agcaaactga aacgttttca tcgctctgga gtgaatacca 660  
 cgacgatttc cggcagtttc tacacatata ttcgcaagat gtggcgtgtt acggtgaaaa 720  
 40 cctggcctat ttccctaaag ggtttattga gaatatgttt ttcgtctcag ccaatccctg 780  
 ggtgagtttc accagttttg atttaaactg ggccaatatg gacaacttct tcgcccccg 840  
 tttcaccatg ggcaaatatt atacgcaagg cgacaagggtg ctgatgccgc tggcgattca 900  
 ggttcacatc gccgtttgtg atggcttcca tgcggcaga atgcttaatg aattacaaca 960  
 gtactgcgat gagtggcagg gcggggcgta attttttttaa ggcagttatt ggtgccctta 1020  
 45 aacgcctggt gctacgcctg aataagtgat aataagcggg tgaatggcag aaattcgtcg 1080  
 aagcttgata tcgaattcct gcagcccggg ggatccacta gttctagagc ggccgccacc 1140  
 gcggtggagc tccagctttt gtcccttta gtgagggtta attgcgcgca ggcctagcta 1200  
 ggtaaagaaa aatacccttg attcttctaa taaccggcg gcccaaatg ccgactcgga 1260  
 gcgaaagata tacctcccc ggggccggga ggtcgcgta ccgaccacgc cgccggccca 1320  
 50 ggcgacgcgc gacacggaca cctgtcccca aaaacgccac catcgagcc acacacggag 1380  
 cgcccggggc cctctggtca accccaggac acacgcggga gcagcgccgg gccggggacg 1440  
 ccctcccggc cgcccggtgc acacgcaggg ggccggcccg tgtctccaga gcgggagccg 1500  
 gaagcatttt cggccggccc ctctacgac cgggacacac gagggaccga aggccggcca 1560

31

```

5  ggcgcga.cct ctcgggccgc acgcgcgctc agggagcgct ctccgactcc gcacggggac 1620
   tcgccagaaa ggatcgtgac ctgcattaat gaatcagggg ataacgcagg aaagaacatg 1680
   tgagcaaaaag gccagcaaaa ggccaggaac cgtaaaaagg ccgcgttgct ggcgtttttc 1740
   cataggcttcc gccccctga cgagcatcac aaaaatcgac gctcaagtca gaggtggcga 1800
   aacccga:cag gactataaag ataccaggcg tttccccctg gaagctccct cgtgcgctct 1860
   cctgtttccga ccctgccgct taccggatac ctgtccgcct ttctcccttc ggggaagcgtg 1920
   gcgctttctc atagctcacg ctgtaggtat ctgagttcgg tgtaggtcgt tcgctccaag 1980
   ctgggctgtg tgcacgaacc ccccgttcag cccgaccgct gcgccttata cggtaactat 2040
   cgtcttgagt ccaacccggt aagacacgac ttatcgccac tggcagcagc cactggtaac 2100
10  aggattagca gagcgaggta tgtaggcggg gctacagagt tcttgaagtg gtggcctaac 2160
   tacggctaca ctagaaggac agtatattggg atctgcgctc tgctgaagcc agttaccttc 2220
   ggaaaaagag ttggtagctc ttgatccggc aaacaaacca ccgctggtag cgggtggtttt 2280
   tttgtttgca agcagcagat tacgcgcaga aaaaaaggat ctcaagaaga tcctttgatc 2340
   ttttctacgg ggtctgacgc tcagtggaaac gaaaactcac gttaagggat tttggtcatg 2400
15  agattat.caa aaaggatctt cacctagatc cttttaaat aaaaatgaag ttttaaatca 2460
   atctaaagta tatatgagta aacttgggtc gacagttacc aatgcttaat cagtgaggca 2520
   cctatct.cag cgatctgtct atttcgttca tccatagttg cctgactccc cgtcgtgtag 2580
   ataactacga tacgggaggg cttaccatct ggccccagtg ctgcaatgat accgcgagac 2640
   ccacgct.cac cggctccaga tttatcagca ataaaccagc cagccggaag ggccgagcgc 2700
20  agaagtggtc ctgcaacttt atccgcctcc atccagtcta ttaattgttg ccgggaagct 2760
   agagtaagta gttcgccagt taatagtttg cgcaacgttg ttgccattgc tacaggcatc 2820
   gtggtgt.cac gtcgctcgtt tggatgggtc tcattcagct ccggttccca acgatcaagg 2880
   cgagttacat gatcccccat gttgtgcaaa aaagcggtta gtccttcggt tcctccgatac 2940
   gttgtcagaa gtaagttggc cgcagtgtta tcactcatgg ttatggcagc actgcataat 3000
25  tctcttactg tcatgccatc cgtaagatgc ttttctgtga ctggtgagta ctcaaccaag 3060
   tcattctgag aatagtgtat gcggcgaccg agttgctctt gcccggcgct aacacgggat 3120
   aataccgcgc cacatagcag aactttaaaa gtgctcatca ttggaaaacg ttcttcgggg 3180
   cgaaaactct caaggatctt accgctgttg agatccagtt cgatgtaacc cactcgtgca 3240
   cccaactgat cttcagcatc ttttactttc accagcgttt ctgggtgagc aaaaacagga 3300
30  aggcaaaaatg ccgcaaaaaa ggggaataagg gcgacacgga aatggtgaat actcatactc 3360
   ttccctttttc aatattattg aagcatttat cagggttatt gtctcatgag cggatacata 3420
   tttgaatgta tttagaaaaa taaacaaaag agtttgtaga aacgcaaaaa ggccatccgt 3480
   caggatggcc ttctgcttaa tttgatgcct ggcagtttat ggccggcgct ctgcccgcga 3540
   ccctccgggc cgttgcttcg caacgttcaa atccgctccc ggccgatttg tcctactcag 3600
35  gagagcggtc accgacaaac aacagataaa acgaaaggcc cagtctttcg actgagcctt 3660
   tcgttttatt tgatgcctgg cagttcccta ctctcgcatg gggagacccc aactaccat 3720
   cggcgctacg gcgtttcact tctgagttcg gcatggggtc aggtgggacc accgcgctac 3780
   tgccgccagg caaattctgt tttatcagac cgcttctgcg ttctgattta atctgtatca 3840
   ggctgaaaat cttctctcat ccgccaaaac agaagctagc ggccgatac 3888
40

```

&lt;210&gt; 21

&lt;211&gt; 4500

&lt;212&gt; DNA

45 &lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pHL3196

50 &lt;400&gt; 21

```

agtagaaaca gggtagataa tcaactcact agtgacatcc acatcgcgag cgcgaaggta 60
cgttctcgag cgcgcgtaat acgactcact atagggcgaa ttgggtacgt tccatcatgg 120
agaaaaaat cactggatat accaccgttg atatatccca atggcatcgt aaagaacatt 180

```



32

	ttgaggcatt	tcagtcagtt	gctcaatgta	cctataacca	gaccgttcag	ctggatatta	240
	cggccttttt	aaagaccgta	aagaaaaata	agcacaagtt	ttatccggcc	tttattcaca	300
	ttcttgcccg	cctgatgaat	gctcatccgg	aattccgtat	ggcaatgaaa	gacggtgagc	360
	tggatgatatg	ggatagtgtt	cacccttggt	acaccgtttt	ccatgagcaa	actgaaacgt	420
5	tttcatcgct	ctggagtga	taccacgacg	atttccggca	gtttctacac	atatattcgc	480
	aagatgtggc	gtgttacggt	gaaaacctgg	cctatttccc	taaagggttt	attgagaata	540
	tgtttttcgt	ctcagccaat	ccctgggtga	gtttcaccag	ttttgattta	aacgtggcca	600
	atatggacaa	cttcttcgcc	cccgttttca	ccatgggcaa	atattatacg	caaggcgaca	660
	aggtgctgat	gccgctggcg	attcagggttc	atcatgccgt	ctgtgatggc	ttccatgtcg	720
10	gcagaatgct	taatgaatta	caacagtact	gcgatgagt	gcagggcggg	gcgcgttaac	780
	gagatcagct	gaaaaatgat	cttcttgaaa	atttgcaggc	cgtacgtgta	ccgggcccc	840
	cctcgactcg	cgaaggagtc	caccatgagt	aaaggagaag	aacttttcac	tggagttgtc	900
	ccaattcttg	ttgaattaga	tgggtgatgtt	aatgggcaca	aattttctgt	cagtggagag	960
	ggtgaagggtg	atgcaacata	cggaaaactt	acccttaaat	ttatttgcac	tactggaaaa	1020
15	ctacctgttc	catggccaac	acttgtcact	actttcactt	atggtgttca	atgcttttca	1080
	agatacccag	atcatatgaa	acagcatgac	tttttcaaga	gtgccatgcc	cgaaggttat	1140
	gtacaggaaa	gaactatatt	tttcaaagat	gacgggaact	acaagacacg	tgctgaagtc	1200
	aagtttgaag	gtgataccct	tgttaataga	atcgagttaa	aagggtattga	ttttaagaa	1260
	gatggaaaca	ttcttgga	caaattggaa	tacaactata	actcacacaa	tgtatacatc	1320
20	atggctgaca	agcagaagaa	cggaaatcaag	gccaaacttca	agaccgcga	caacatcgag	1380
	gacggcggcg	tgcagctggc	cgaccactac	cagcagaaca	ccccaatgg	cgatggccct	1440
	gtccttttac	cagacaacca	ttactgtcc	acacaatctg	ccctttcgaa	agatcccaac	1500
	gaaaagagag	accacatggt	ccttcttgag	tttgaacag	ctgctgggat	tacacatggc	1560
	atggatgaac	tatacaaggg	atcccatcac	catcaccatc	actaagctcc	atggtctaga	1620
25	tatcgatagg	cctagctagg	taaagaaaaa	tacccttggt	tctactaata	acccggcggc	1680
	ccaaaatgcc	gactcggagc	gaaagatata	cctcccccg	ggccgggagg	tcgcgtcacc	1740
	gaccacgcg	ccggcccagg	cgacgcgcga	cacggacacc	tgtcccaaaa	aacgccacca	1800
	tcgcagccac	acacggagcg	cccggggccc	tctggtcaac	cccaggacac	acgcgggagc	1860
	agcgcggggc	cggggacgcc	ctcccggccg	ccggtgccac	acgcaggggg	ccggcccgtg	1920
30	tctccagagc	gggagccgga	agcattttcg	gccggccctt	cctacgaccg	ggacacacga	1980
	gggaccgaag	gccggccagg	cgcgacctct	cgggccgcac	gcgcgctcag	ggagcgctct	2040
	ccgactccgc	acggggactc	gccagaaagg	atcgtgacct	gcattaatga	atcaggggat	2100
	aacgcaggaa	agaacatgtg	agcaaaaggc	cagcaaaagg	ccaggaaccg	taaaaaggcc	2160
	gcgttgctgg	cgtttttcca	taggtccgc	ccccctgacg	agcatcacia	aaatcgacgc	2220
35	tcaagtcaga	ggtggcgaaa	cccgacagga	ctataaagat	accaggcggt	tccccctgga	2280
	agctccctcg	tgcgctctcc	tgttccgacc	ctgccgctta	ccggatacct	gtccgccttt	2340
	ctcccttcgg	gaagcgtggc	gctttctcat	agctcacgct	gtaggtatct	cagttcgggtg	2400
	taggtcgttc	gtccaagct	gggctgtgtg	cacgaacccc	ccgttcagcc	cgaccgctgc	2460
	gccttatccg	gtaactatcg	tcttgagtcc	aaccgggtaa	gacacgactt	atcgccactg	2520
40	gcagcagcca	ctggtaacag	gattagcaga	gcgaggatat	taggcgggtg	tacagagttc	2580
	ttgaagtgg	ggcctaacta	cggctacact	agaaggacag	tatttgggtat	ctgcgctctg	2640
	ctgaagccag	ttaccttcgg	aaaaagagtt	ggtagctctt	gatccggcaa	acaaaccacc	2700
	gctggtagcg	gtggtttttt	tgtttgcaag	cagcagatta	cgcgcagaaa	aaaaggatct	2760
	caagaagatc	ctttgatctt	ttctacgggg	tctgacgctc	agtggaaacga	aaactcacgt	2820
45	taaggcattt	tggatcatgag	attatcaaaa	aggatcttca	cctagatcct	tttaaatata	2880
	aaatgaagtt	ttaaatcaat	ctaaagtata	tatgagtaaa	cttgggtctga	cagttacca	2940
	tgcttaataca	gtgaggcacc	tatctcagcg	atctgtctat	ttcgttcatc	catagttgcc	3000
	tgactccccg	tcgtgtagat	aactacgata	cgggagggtt	taccatctgg	ccccagtgtc	3060
	gcaatgatac	cgcgagaccc	acgtccaccg	gctccagatt	tatcagcaat	aaaccagcca	3120
50	gccggaagg	ccgagcgcag	aagtggctct	gcaactttat	ccgcctccat	ccagtcctatt	3180
	aattgttgcc	gggaagctag	agtaagtagt	tcgccagtta	atagtttgcg	caacgttggt	3240
	gccattgcta	caggcatcgt	ggtgtcacgc	tcgtcgtttg	gtatggcttc	attcagctcc	3300
	ggttcccaac	gatcaaggcg	agttacatga	tcccccatgt	tgtgcaaaaa	agcgggttagc	3360

33

```

5  tccttcggtc ctccgatcgt tgtcagaagt aagttggccg cagtgttatc actcatgggt 3420
   atggcagcac tgcataattc tcttactgtc atgccatccg taagatgctt ttctgtgact 3480
   ggtgagtact caaccaagtc attctgagaa tagtgtatgc ggcgaccgag ttgctcttgc 3540
   ccggcgt:caa cacgggataa taccgcgcca catagcagaa ctttaaaagt gctcatcatt 3600
   ggaaaacggt cttcggggcg aaaactctca aggatcttac cgctgttgag atccagttcg 3660
   atgtaaccca ctcgtgcacc caactgatct tcagcatctt ttactttcac cagcgtttct 3720
   ggggtgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg gaataagggc gacacggaaa 3780
   tgttgaatac tcatactctt cctttttcaa tattattgaa gcatttatca gggttatctgt 3840
   ctcatgagcg gatacatatt tgaatgtatt tagaaaaata aacaaaagag tttgtagaaa 3900
10  cgcaaaaagg ccatccgtca ggatggcctt ctgcttaatt tgatgcctgg cagtttatgg 3960
   cgggcgt:cct gcccgccacc ctccgggccc ttgcttcgca acgttcaa at ccgctcccgg 4020
   cggatttgtc ctactcagga gagcgttcac cgacaaacaa cagataaaac gaaaggccca 4080
   gtcttttcgac tgagcctttc gttttatttg atgcctggca gttccctact ctcgcatggg 4140
   gagaccccac actaccatcg gcgctacggc gtttccactc tgagttcggc atgggggtcag 4200
15  gtgggacacac cgcgctactg ccgccaggca aattctgttt tatcagaccg cttctgcgtt 4260
   ctgattta at ctgtatcagg ctgaaaatct tctctcatcc gccaaaacag aagctagcgg 4320
   ccgatcccca aaaaaaaaaa aaaaaaaaaa aaaaagagtc cagagtggcc ccgccgttcc 4380
   gcgcccgggg gggggggggg gggggacact ttccggacatc tggtcgacct ccagcatcgg 4440
   gggaaaaaaa aaaaacaaag tttcgcgccg agtactggtc gacctccgaa gttggggggg 4500

```

&lt;210&gt; 22

&lt;211&gt; 4721

&lt;212&gt; DNA

25 &lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pHL3224

30 &lt;400&gt; 22

```

   atctagacca tggagcttag tgatgggtgat ggtgatggga tcccttgat agttcatcca 60
   tgccatgtgt aatcccagca gctgttacaa actcaagaag gaccatgtgg tctctctttt 120
   cgttgggatac ttccgaaagg gcagattgtg tggacaggta atggttgtct ggtaaaagga 180
   cagggccatc gccaatggg gtgttctgct ggtagtggtc ggccagctgc acgcccgct 240
35  cctcgatgtt gtggcgggtc ttgaagttgg ccttgattcc gttcttctgc ttgtcagcca 300
   tgatgtatac attgtgtgag ttatagttgt attccaattt gtgtccaaga atgtttccat 360
   cttcttttaa atcaatacct tttaactcga ttctattaac aagggtatca ctttcaaact 420
   tgaacttcagc acgtgtcttg tagttcccgat catctttgaa aaatatagtt ctttccctgta 480
   cataaccttc gggcatggca ctcttgaaaa agtcatgctg tttcatatga tctgggtatc 540
40  ttgaaaagca ttgaacacca taagtgaag tagtgacaag tgttggccat ggaacaggta 600
   gttttccagt agtgcaaata aatttaaggg taagttttcc gtatgttgca tcaccttcac 660
   cctctccact gacagaaaat ttgtgcccac taacatcacc atctaattca acaagaattg 720
   ggacaactcc agtgaaaagt tcttctcctt tactcatggg ggactccttc gcgagtcgag 780
   gggggggccc gtacacgtac gcgctcgaga acgtaccttc gcgctcgaga tgtggatgtc 840
45  actcagttag tgattatcta cctgttttct actccccccc aacttcggag gtcgaccagt 900
   actccgggcg aaactttgtt tttttttttt cccccgatgc tggaggtcga ccagatgtcc 960
   gaaagtgtcc cccccccccc cccccccgg cgcggaacgg cggggccact ctggactott 1020
   tttttttttt tttttttttt ttttggggat cggccgctag cttctgtttt ggcggatgag 1080
   agaagatttt cagcctgata cagattaaat cagaacgcag aagcgggtctg ataaaacaga 1140
50  atttgcctgg cggcagtagc gcggtgggtc cacctgaccc catgccgaac tcagaagtga 1200
   aacgccgtag cgccgatggg agtgtggggg ctccccatgc gagagtaggg aactgccagg 1260
   catcaaaata aacgaaaggc tcagtcgaaa gactgggcct ttcgtttttat ctgttgtttg 1320
   tcggtgaacg ctctcctgag taggacaaat ccgccgggag cggatttgaa cgttgcgaag 1380

```

	caacggcccg	gaggggtggcg	ggcaggacgc	ccgccataaa	ctgccaggca	tcaaattaag	1440
	cagaaggcca	tcttgacgga	tggccttttt	gcgtttctac	aaactctttt	gtttattttt	1500
	ctaaatacat	tcaaataatgt	atccgctcat	gagacaataa	ccctgataaa	tgcttcaata	1560
	atattgaaaa	aggaagagta	tgagtattca	acatttccgt	gtcgccctta	ttcccttttt	1620
5	tgcggcattt	tgcccttctg	tttttgctca	cccagaaaacg	ctgggtgaaag	taaaagatgc	1680
	tgaagatcag	ttgggtgcac	gagtggttga	catcgaactg	gatctcaaca	gcggtaagat	1740
	ccttgagagt	tttcgccccg	aagaacgttt	tccaatgatg	agcactttta	aagttctgct	1800
	atgtggcgcg	gtattatccc	gtgttgacgc	cgggcaagag	caactcggtc	gccgcattca	1860
	ctattctcag	aatgacttgg	ttgagtactc	accagtcaca	gaaaagcatc	ttacggatgg	1920
10	catgacagta	agagaattat	gcagtgctgc	cataaccatg	agtgataaca	ctgcggccaa	1980
	cttacttctg	acaacgatcg	gaggaccgaa	ggagctaacc	gcttttttgc	acaacatggg	2040
	ggatcatgta	actcgcttg	atcgttgga	accggagctg	aatgaagcca	taccaaacga	2100
	cgagcgtgac	accacgatgc	ctgtagcaat	ggcaacaacg	ttgcgcaaac	tattaactgg	2160
	cgaactactt	actctagctt	cccggcaaca	attaatagac	tggatggagg	cggataaagt	2220
15	tgcaggacca	cttctgcgct	cggcccttcc	ggctggctgg	tttattgctg	ataaatctgg	2280
	agccggtgag	cgtgggtctc	gcggtatcat	tgcagcactg	gggccagatg	gtaagccctc	2340
	ccgtatcgta	gttatctaca	cgacggggag	tcaggcaact	atggatgaac	gaaatagaca	2400
	gatcgctgag	ataggtgcct	cactgattaa	gcattggtaa	ctgtcagacc	aagtttactc	2460
	atatatactt	tagattgatt	taaaacttca	tttttaattt	aaaaggatct	aggtgaagat	2520
20	cctttttgat	aatctcatga	ccaaaatccc	ttaacgtgag	tttctgcttc	actgagcgtc	2580
	agaccccgta	gaaaagatca	aaggatcttc	ttgagatcct	ttttttctgc	gcgtaatctg	2640
	ctgcttgcaa	acaaaaaac	caccgctacc	agcggtggtt	tgtttgccgg	atcaagagct	2700
	accaactctt	tttccgaagg	taactggctt	cagcagagcg	cagataccaa	atactgtcct	2760
	tctagtgtag	ccgtagttag	gccaccactt	caagaactct	gtagcaccgc	ctacatacct	2820
25	cgctctgcta	atcctgttac	cagtggctgc	tgccagtggc	gataagtcgt	gtcttaccgg	2880
	gttggactca	agacgatagt	taccggataa	ggcgagcg	tcgggctgaa	cgggggggtt	2940
	gtgcacacag	cccagcttgg	agcgaacgac	ctacaccgaa	ctgagatacc	tacagcgtga	3000
	gctatgagaa	agcgccacgc	ttcccgaagg	gagaaaggcg	gacaggatc	cggtaagcgg	3060
	cagggtcgga	acaggagagc	gcacgaggga	gcttccagg	ggaaacgcct	ggatatcttta	3120
30	tagtcctgtc	gggtttcgcc	acctctgact	tgagcgtcga	tttttgtgat	gctcgtcagg	3180
	ggggcgagc	ctatggaaaa	acgccagcaa	cgcggccttt	ttacgggttc	tgcccttttg	3240
	ctggcctttt	gtccacatgt	tctttcctgc	gttatccct	gattcattaa	tgcaggtcac	3300
	gatcctttct	ggcgagtccc	cgtgcggagt	cggagagcgc	tccctgagcg	cgcgtgcggc	3360
	ccgagaggtc	gcgcctggcc	ggccttcggt	ccctcgtgtg	tcccggctgt	aggagggggc	3420
35	ggccgaaaat	gcttccggct	cccgtctg	agacacgggc	cggcccccctg	cgtgtggcac	3480
	gggcggcccg	gagggcgctc	ccggcccggc	gctgctccc	cgtgtgtcct	ggggttgacc	3540
	agagggccccc	gggcgctccg	tgtgtggctg	cgatgggtggc	gtttttgggg	acaggtgtcc	3600
	gtgtcgcgcg	tcgcctgggc	cggcgcgctg	gtcggtgacg	cgacctcccg	gccccggggg	3660
	aggtatatct	ttcgctccga	gtcggcattt	tgggcccgcg	ggttattagt	agaaacaagg	3720
40	gtatttttct	ttacctagct	aggcctgcgc	gcaattaacc	ctcactaaag	ggaacaaaag	3780
	ctggagctcc	accgcggtgg	cggccgctct	agaactagt	gatcccccg	gctgcaggaa	3840
	ttcgatatca	agcttcgacg	aattttctgcc	attcatccgc	ttattatcac	ttattcaggc	3900
	gtagcacacag	gcgtttaagg	gcaccaataa	ctgccttaaa	aaaattacgc	cccgccttgc	3960
	cactcatcgc	agtactgttg	taattcatta	agcattctgc	cgacatggaa	gccatcacaa	4020
45	acggcatgat	gaacctgaat	cgccagcggc	atcagcacct	tgtcgccctg	cgtataatat	4080
	ttgcccatgg	tgaaaacggg	ggcgaagaag	ttgtccatat	tggccacggt	taaatcaaaa	4140
	ctggtgaaac	tcaccaggg	attggctgag	acgaaaaaca	tattctcaat	aaacccttta	4200
	gggaaatagg	ccaggttttc	accgtaacac	gccacatctt	gcgaatatat	gtgtagaaac	4260
	tgccggaaat	cgtcgtggta	ttcaactccag	agcgatgaaa	acgtttcagt	ttgctcatgg	4320
50	aaaacggtgt	aacaaggggtg	aacactatcc	catatcacca	gctcaccgtc	tttcattgcc	4380
	atacgggaatt	ccggatgagc	attcatcagg	cgggcaagaa	tgtgaataaa	ggccggataa	4440
	aacttgtgct	tatttttctt	tacgggtctt	aaaaaggccg	taatatccag	ctgaacggtc	4500
	tggttatagg	tacattgagc	aactgactga	aatgcctcaa	aatgttcttt	acgatgccat	4560

35

```

tgggatatat caacggtggt atatccagtg atttttttct ccatttttagc ttccttagct 4620
cctgaaaatc tcgtcgaagc ttatcgatac cgtcgacctc gagggggggc ccggtacggc 4680
ctgcaaattt tcaagaagat cttttttcag ctgatctcgt t 4721

```

5

&lt;210&gt; 23

&lt;211&gt; 5517

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

10

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pHL3235

&lt;400&gt; 23

```

15 agtagaaaca gggtagataa tcaactcactg agtgacatcc acatcgcgag cgogaaggta 60
   cgttctcagag cgcgcgtaat acgactcact atagggcgaa ttgggtacgt tccatcatgg 120
   agaaaaaaat cactggatat accaccgttg atatatccca atggcatcgt aaagaacatt 180
   ttgaggcatt tcagtcagtt gctcaatgta cctataacca gaccgttcag ctggatatta 240
   cggccttttt aaagaccgta aagaaaaata agcacaagtt ttatccggcc tttattcaca 300
20 ttcttgcccg cctgatgaat gctcatccgg aattccgtat ggcaatgaaa gacggtgagc 360
   tggatgatag ggatagtgtt cacccttggt acaccgtttt ccatgagcaa actgaaacgt 420
   tttcatcgct ctggagtga taccacgacg atttccggca gtttctacac atatattcgc 480
   aagatgtggc gtgttacggg gaaaacctgg cctatttccc taaagggttt attgagaata 540
   tgtttttcgt ctacgccaat ccctgggtga gtttcaccag ttttgattta aacgtggcca 600
25 atatggacaa cttcttcgcc ccggttttca ccatgggcaa atattatacg caaggcgaca 660
   aggtgctgat gccgctggcg attcaggttc atcatgccgt ctgtgatggc tccatgtcgc 720
   gcagaatgct taatgaatta caacagtact gcgatgagtg gcagggcggg gcgcttaac 780
   gagatcagct gaaaaatgat cttcttgaaa atttgcaggc cgtacgtgta ccgggcccc 840
   cctcgactcg cgaaggagtc caccatgagt aaaggagaag aacttttcac tggagtgtgc 900
30 ccaattcttg ttgaattaga tggatgatgtt aatgggcaca aattttctgt cagtggagag 960
   ggtgaagggtg atgcaacata cggaaaactt acccttaaat ttatttgac tactggaaaa 1020
   ctacctgttc catggccaac acttgtcact actttcactt atggtgttca atgcttttca 1080
   agatacccag atcatatgaa acagcatgac tttttcaaga gtgccatgcc cgaaggttat 1140
   gtacaggaaa gaactatatt tttaaagat gacgggaact acaagacacg tgctgaagtc 1200
35 aagtttgaag gtgataccct tgttaataga atcgagttaa aaggatttga ttttaaagaa 1260
   gatggaaaca ttcttgga caaattggaa tacaactata actcacacaa tgtatacatc 1320
   atggctgaca agcagaagaa cggaatcaag gccaaacttca agaccgcga caacatcgag 1380
   gacggcgggc tgcagctggc cgaccactac cagcagaaca cccaattgg cgatggccct 1440
   gtctttttac cagacaacca ttacctgtcc acacaatctg ccttttcgaa agatcccaac 1500
40 gaaaagagag accacatggg cttcttgag tttgtaacag ctgctgggat tacacatggc 1560
   atggatgaac tatacaaggg atcttcatga tctcagcaaa ctcttccttc ttaatccttc 1620
   cagactcgaa gtcaattcgt gcatcaatcc gggccctaga caccatggcc tccaccatac 1680
   tggaaattcc aactggtcct ctgtatgagc tgctagggaa gaatttctcg aataggttgc 1740
   aacacttctg gtacatttgt tcatcctcaa ggattccctt ttgactcgta ttgagaatgg 1800
45 aacggtttct cttagggtac caagagtgtg tagttgccac agcatcatat tccatgcttt 1860
   tggctggacc atgggctggc attaccgcag cattgtttac agattcaatt tcttatgac 1920
   tgacaaacgg gttcatggga ttacaaagtc ttccctgata gtcttcatcc attagtctcc 1980
   atttcaggca aacttccggg atgtggagat tccgaatgtt gtacagggtt ggtccgcgat 2040
   ctgaaaccaa cagtcttgc tttgagcggg tctgtctcca cagcttcttt agctcgaatg 2100
50 acctcctcgt ttggatttgt gtgtctcccc tgtgacaccg gtatgtatat ctgtagtctc 2160
   tgatgaataa ttggagagcc atttgggctg ttgccggtcc aagatcattg tttatcatgt 2220
   tattctttat cactgttact ccaatgctca tatcagccga ttcattaatt cctgatactc 2280
   caaagctggg caactccata ctaaaattgg ctacaaatcc atagcggtag aaaaagcttg 2340

```

36

	tgaattcgaa	tggttcctgtc	ctattttatat	aggactttttt	cttgetcata	ttgatcccaa	2400
	ctagcttgca	ggttctgtag	aatctatcca	ctcccgttg	tattccctca	tgatttggtg	2460
	cattcacgat	gagagcaaaa	tcatcagagg	actgaagtcc	atcccaccag	tatgtgggtt	2520
	tggtgtatct	cttttgccca	agattcagga	ttgagactcc	caacactgta	ctcagcatgt	2580
5	tgaacatacc	catcatcatt	cccgggctta	atgaggctgt	gccgtctatt	atgagaggat	2640
	cgataggcct	agctaggtaa	agaaaaatac	ccttgtttct	actaataacc	cggcggccca	2700
	aaatgccgac	tcggagcgaa	agatatacct	cccccggggc	cgggaggtcg	cgtcaccgac	2760
	cacgccgccc	gcccaggcga	cgcgcgacac	ggacacctgt	ccccaaaaac	gccaccatcg	2820
	cagccacaca	cggagcgccc	ggggccctct	ggtcaacccc	aggacacacg	cgggagcagc	2880
10	gccgggccc	ggacgccttc	cgggccgccc	gtgccacacg	cagggggccg	gcccgtgtct	2940
	ccagagcggg	agccggaagc	attttcggcc	ggccctctct	acgaccggga	cacacgaggg	3000
	accgaaggcc	ggccaggcgc	gacctctcgg	gccgcacgcg	cgctcaggga	gcgctctccg	3060
	actccgcacg	gggactcgcc	agaaaggatc	gtgacctgca	ttaatgaatc	aggggataac	3120
	gcaggaaaga	acatgtgagc	aaaaggccag	caaaaggcca	ggaaccgtaa	aaaggccgcg	3180
15	ttgctggcgt	ttttccatag	gctccgcccc	cctgacgagc	atcacaaaaa	tcgacgctca	3240
	agtcagaggt	ggcgaaaccc	gacaggacta	taaagatacc	aggcgtttcc	ccctggaagc	3300
	tccctcgtgc	gctctcctgt	tccgacctgt	ccgcttaccg	gatacctgtc	cgctttcttc	3360
	ccttcgggaa	gcgtggcgct	ttctcatagc	tcacgctgta	ggtatctcag	ttcgggtgtg	3420
	gtcgttcgct	ccaagctggg	ctgtgtgcac	gaaccccccg	ttcagcccca	ccgctgcgcc	3480
20	ttatccggta	actatcgtct	tgagtccaac	ccggtgaagc	acgacttatc	gccactggca	3540
	gcagccactg	gtaacaggat	tagcagagcg	aggatgtgag	gcggtgctac	agagttcttg	3600
	aagtgggtggc	ctaactacgg	ctacactaga	aggacagtat	ttgggtatctg	cgctctgctg	3660
	aagccagtta	ccttcggaaa	aagagttggt	agctcttgat	ccggcaaaaca	aaccaccgct	3720
	ggtagcgggtg	gttttttttgt	ttgcaagcag	cagattacgc	gcagaaaaaa	aggatctcaa	3780
25	gaagatcctt	tgatcttttc	tacgggggtct	gacgctcagt	ggaacgaaaa	ctcacgttaa	3840
	gggatttttg	tcatgagatt	atcaaaaagg	atcttcacct	agatcctttt	aaattaaaaa	3900
	tgaagtttta	aatcaatcta	aagtatatat	gagtaaactt	ggtctgacag	ttaccaatgc	3960
	ttaatcagtg	aggcacctat	ctcagcgatc	tgtctatttc	gttcatccat	agttgcctga	4020
	ctccccgtcg	tgtagataac	tacgatacgg	gagggcttac	catctggccc	cagtgtcgca	4080
30	atgataccgc	gagacccacg	ctcaccggct	ccagatttat	cagcaataaa	ccagccagcc	4140
	ggaagggccg	agcgcagaag	tggtcctgca	actttatccg	cctccatcca	gtctattaat	4200
	tgttgcgggg	aagctagagt	aagtagttcg	ccagttaata	gtttgcgcaa	cgttggtgcc	4260
	attgctacag	gcacgtgggt	gtcacgctcg	togtttggtg	tggtttcatt	cagctccggg	4320
	tcccaacgat	caaggcgagt	tacatgatcc	cccatgttgt	gcaaaaaagc	ggttagctcc	4380
35	ttcgggtctc	cgatcgttgt	cagaagtaag	ttggccgcag	tgttatcact	catggttatg	4440
	gcagcactgc	ataattctct	tactgtcatg	ccatccgtaa	gatgcttttc	tgtgactggg	4500
	gagtactcaa	ccaagtcatt	ctgagaatag	tgtatgcggc	gaccgagttg	ctcttgcccg	4560
	gcgtcaacac	gggataatac	cgcgccacat	agcagaactt	taaaagtgtc	catcattgga	4620
	aaacgttctt	cggggcgaaa	actctcaagg	atcttaccgc	tgttgagatc	cagttcgatg	4680
40	taaccacttc	gtgcacccaa	ctgatcttca	gcacctttta	ctttcaccag	cgtttctggg	4740
	tgagcaaaaa	caggaaggca	aaatgccgca	aaaaagggaa	taagggcgac	acggaaatgt	4800
	tgaataactca	tactcttctc	ttttcaatat	tattgaagca	tttatcaggg	ttattgtctc	4860
	atgagcggat	acatatttga	atgtattttag	aaaaataaac	aaaagagttt	gtagaaacgc	4920
	aaaaaggcca	tccgtcagga	tggccttctg	cttaatttga	tgcttggcag	tttatggcgg	4980
45	gcgtcctgcc	cgccaccctc	cgggcccgtt	cttcgcaacg	ttcaaaccg	ctcccggcgg	5040
	atttgctcta	ctcaggagag	cgttcaccga	caaacaacag	ataaaacgaa	aggcccagtc	5100
	tttcgactga	gcctttcggt	ttatttgatg	cctggcagtt	ccctactctc	gcattggggg	5160
	acccacact	accatcggcg	ctacggcggt	tcacttctga	gttcggcatg	gggtcagggtg	5220
	ggaccaccgc	gctactgccg	ccaggcaaat	tctgttttat	cagaccgctt	ctgcgttctg	5280
50	attttaatctg	tatcaggctg	aaaatcttct	ctcatccgcc	aaaacagaag	ctagcggccc	5340
	atccccaaaa	aaaaaaaaaa	aaaaaaaaaa	aagagtccag	agtggccccg	ccgttccgcg	5400
	ccgggggggg	gggggggggg	ggacactttc	ggacatctgg	tcgacctcca	gcacgggggg	5460
	aaaaaaaaaa	aacaaagttt	cgcccggagt	actggtcgac	ctccgaagtt	gggggggg	5517

&lt;210&gt; 24

&lt;211&gt; 5699

5 &lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pHL3236

10

&lt;400&gt; 24

```

cctctcataa tagacggcac agcctcatta agcccgggaa tgatgatggg tatgttcaac 60
atgctgagta cagtgttggg agtctcaatc ctgaatcttg ggcaaaagag atacaccaa 120
accacatact ggtgggatgg acttcagtc tctgatgatt ttgctctcat cgtgaatgca 180
15 ccaaatactg agggaaataca agcgggagtg gatagattct acagaacctg caagctagtt 240
gggatcaata tgagcaagaa aaagtcctat ataaatagga caggaacatt cgaattcaca 300
agctttttct accgctatgg atttgtagcc aatttttagta tggagttgcc cagctttgga 360
gtatcaggaa ttaatgaatc ggctgatatg agcattggag taacagtgat aaagaataac 420
atgataaaca atgatcttgg accggcaaca gcccaaattg ctctccaatt attcatcaag 480
20 gactacagat atacataacc gtgtcacagg ggagacacac aaatccaaac gaggaggtca 540
ttcgagctaa agaagctgtg ggagcagacc cgctcaaagg caggactgtt ggtttcagat 600
ggcggaccaaa acctgtacaa cattcggaat ctccacatcc cggaagtttg cctgaaatgg 660
gaactaatgg atgaagacta tcagggaaga ctttgtaatc ccatgaaccc gtttgtcagt 720
cataaggaaa ttgaatctgt aaacaatgct gcggtaatgc cagcccatgg tccagccaaa 780
25 agcatggaat atgatgctgt ggcaactaca cactcttggg tccctaagag aaaccgttcc 840
attctcaata cgagtcaaag gggaaatcctt gaggatgaac aaatgtacca gaagtgttgc 900
aacctat.tcg agaaattctt ccctagcagc tcatacagaa gaccagttgg aatttccagt 960
atggtggagg ccatggtgtc tagggcccgg attgatgcac gaattgactt cgagtctgga 1020
aggattaaga aggaagagtt tgctgagatc atgaagatcc cccgggctgc aggaattcga 1080
30 tatcaagctt cgacgaattt ctgccattca tccgcttatt atcacttatt caggcgtagc 1140
accaggcgtt taagggcacc aataactgcc ttaaaaaaat tacgccccgc cctgccactc 1200
atcgcgatc tgttgtaatt cattaagcat tctgcgcaca tggaagccat caciaacggc 1260
atgatgaacc tgaatcgcca gcggcatcag caccttgctg ccttgcgat aatatttgcc 1320
catggtgaaa acggggggcga agaagttgtc catattggcc acgtttaaat caaaactggg 1380
35 gaaactcacc cagggattgg ctgagacgaa aaacatattc tcaataaacc ctttagggaa 1440
ataggccagg ttttcaccgt aacacgccac atcttgcgaa tatatgtgta gaaactgccg 1500
gaaatcgctc tggatttcac tccagagcga tgaaaacgtt tcagtttgct catggaaaac 1560
ggtgtaacaa ggggtgaacac tatcccatat caccagctca ccgtctttca ttgccatacg 1620
gaattccgga tgagcattca tcaggcgggc aagaatgtga ataaaggccg gataaaactt 1680
40 gtgcttattt ttctttacgg tctttaaaaa ggccgtaata tccagctgaa cggctctggt 1740
ataggtacat tgagcaactg actgaaatgc ctcaaatgt tctttacgat gccattggga 1800
tatatcaacg gtggtatatc cagtgatatt tttctccatt ttagcttcct tagctcctga 1860
aaatctcgtc gaagcttatc gataccgtcg acctcgaggg ggggcccggg acggcctgca 1920
aattttcaag aagatcattt ttcagctgat ctcgttatct agaccatgga gcttagtgat 1980
45 ggtgatgggt atgggatccc ttgtatagtt catccatgcc atgtgtaatc ccagcagctg 2040
ttacaaactc aagaaggacc atgtggtctc tcttttcgtt gggatctttc gaaagggcag 2100
attgtgtgga caggtaatgg ttgtctggta aaaggacagg gccatcgcca attgggggtg 2160
tctgctggta gtggtcggcc agctgcacgc cgccgtctc gatgttggtg cgggtcttga 2220
agttggcett gattccgttc ttctgcttgt cagccatgat gtatacattg tgtgagttat 2280
50 agttgtattc caatttgtgt ccaagaatgt ttccatcttc tttaaaatca atacctttta 2340
actcgattct attacaagg gtatcacctt caaacttgac ttcagcacgt gtctttagt 2400
tcccgtcac tttgaaaaat atagttcttt cctgtacata accttcgggc atggcactct 2460
tgaaaaagtc atgctgtttc atatgatctg ggtatcttga aaagcattga acaccataag 2520

```

	tgaaagtagt	gacaagtgtt	ggccatggaa	caggtagttt	tccagtagtg	caaataaatt	2580
	taagggtaag	ttttccgtat	gttgcacac	cttcaccctc	tccactgaca	gaaaatttgt	2640
	gcccatcaac	atcaccatct	aattcaacaa	gaattgggac	aactccagtg	aaaagttctt	2700
	ctccttact	catggtggac	tccttcgcga	gtcgagggg	ggcccggtac	acgtacgcgc	2760
5	tgcgaaacgt	accttcgcgc	tcgcgatgtg	gatgtcactc	agtgagtgat	tatctaccct	2820
	gtttctactc	ccccccaact	tcggaggctg	accagtactc	cgggcgaaac	tttggtttttt	2880
	ttttttcccc	cgatgctgga	ggtcgaccag	atgtccgaaa	gtgtcccccc	cccccccccc	2940
	ccccggcgcg	gaacggcggg	gccactctgg	actctttttt	tttttttttt	tttttttttt	3000
	ggggatcggc	cgctagcttc	tgttttggcg	gatgagagaa	gattttcagc	ctgatacaga	3060
10	ttaaatcaga	acgcagaagc	ggtctgataa	aacagaattt	gcctggcggc	agtagcgcg	3120
	tgggtcccacc	tgaccccatg	ccgaactcag	aagtgaacg	ccgtagcgcc	gatggtagtg	3180
	tgggggtctcc	ccatgcgaga	gtagggaact	gccaggcatc	aaataaaaacg	aaaggctcag	3240
	tcgaaagact	gggcctttcg	ttttatctgt	tgtttgtcgg	tgaacgctct	cctgagtagg	3300
	acaaatccgc	cgggagcgga	tttgaacggt	gcgaagcaac	ggcccggagg	gtggcgggca	3360
15	ggacgcccgc	cataaactgc	caggcatcaa	attaagcaga	aggccatcct	gacggatggc	3420
	ctttttgcgt	ttctacaaac	tcttttggtt	atattttctaa	atacattcaa	atatgtatcc	3480
	gctcatgaga	caataaccct	gataaatgct	tcaataatat	tgaaaaagga	agagtatgag	3540
	tattcaacat	ttccgtgtcg	cccttattcc	cttttttgcg	gcattttgce	ttcctgtttt	3600
	tgctcaccga	gaaacgctgg	tgaaagtaaa	agatgctgaa	gatcagttgg	gtgcacgagt	3660
20	gggttacatc	gaactggatc	tcaacagcgg	taagatcctt	gagagttttc	gccccgaaga	3720
	acgtttttcca	atgatgagca	cttttaaagt	tctgctatgt	ggcgcggtat	tatcccgtgt	3780
	tgacgcgcgg	caagagcaac	tcggtcgccg	catacactat	tctcagaatg	acttggttga	3840
	gtactcacca	gtcacagaaa	agcatcttac	ggatggcatg	acagtaagag	aattatgcag	3900
	tgctgccata	accatgagtg	ataacactgc	ggccaactta	cttctgacaa	cgatcggagg	3960
25	accgaaggag	ctaaccgctt	ttttgcacaa	catgggggat	catgtaactc	gccttgatcg	4020
	ttgggaaccg	gagctgaatg	aagccatacc	aaacgacgag	cgtgacacca	cgatgcctgt	4080
	agcaatggca	acaacgttgc	gcaaactatt	aactggcgaa	ctaactactc	tagcttcccc	4140
	gcaacaatta	atagactgga	tggaggcgga	taaagttgca	ggaccacttc	tgcgctcggc	4200
	ccttccggct	ggctgggtta	ttgctgataa	atctggagcc	ggtgagcgtg	ggtctcgcg	4260
30	tatcattgca	gcaactgggc	cagatggtaa	gccctcccg	atcgtagtta	tctacacgac	4320
	ggggagtcag	gcaactatgg	atgaacgaaa	tagacagatc	gctgagatag	gtgcctcact	4380
	gattaagcat	tggttaactgt	cagaccaagt	ttactcatat	atactttaga	ttgatttaaa	4440
	acttcatttt	taatttaaaa	ggatctaggt	gaagatcctt	tttgataatc	tcatgaccaa	4500
	aatcccttaa	cgtgagtttt	cgttccactg	agcgtcagac	cccgtagaaa	agatcaaagg	4560
35	atcttcttga	gatccttttt	ttctgcgcgt	aatctgctgc	ttgcaaacaa	aaaaaccacc	4620
	gctaccagcg	gtgggtttgt	tgccggatca	agagctacca	actctttttc	cgaaggtaac	4680
	tggcttcagc	agagcgcaga	taccaaatac	tgtccttcta	gtgtagccgt	agttaggcca	4740
	ccacttcaag	aactctgtag	caccgcctac	atacctcgct	ctgctaatac	tgttaccagt	4800
	ggctgctgcc	agtggcgata	agtcgtgtct	taccgggttg	gactcaagac	gatagttacc	4860
40	ggataagcg	cagcggtcgg	gctgaacggg	gggttcgtgc	acacagccca	gcttgagcgc	4920
	aacgacctac	accgaactga	gatacctaca	gcgtgagcta	tgagaaagcg	ccacgcttcc	4980
	cgaaggggaga	aaggcggaca	ggtatccggt	aagcggcagg	gtcggaacag	gagagcgcac	5040
	gagggagctt	ccagggggaa	acgcctggta	tctttatagt	cctgtcgggt	ttcgccacct	5100
	ctgacttgag	cgtcgatttt	tgtgatgctc	gtcagggggg	cggagcctat	ggaaaaacgc	5160
45	cagcaacgcg	gccttttttac	ggttcctggc	cttttgctgg	ccttttgctc	acatgttctt	5220
	tcttgcttta	tcccttgatt	cattaatgca	ggtcacgata	ctttctggcg	agtccccgtg	5280
	cggagtcgga	gagcgcctcc	tgagcgcgcg	tgcggcccca	gaggtcgcgc	ctggccggcc	5340
	ttcggtccct	cgtgtgtccc	ggtcgtagga	ggggccggcc	gaaaaatgctt	ccggtcccg	5400
	ctctggagac	acgggcccgc	cccctgcgtg	tggcacgggc	ggccggggagg	gcgtcccccg	5460
50	cccggcgctg	ctcccgcgtg	tgtcctgggg	ttgaccagag	ggccccgggc	gctccgtgtg	5520
	tggctgcgat	ggtggcggtt	ttggggacag	gtgtccgtgt	cgcgcgtcgc	ctgggcccgc	5580
	ggcgtggctg	gtgacgcgac	ctcccggccc	cgggggaggt	atatctttcg	ctccgagtcg	5640
	gcattttggg	ccgcggggtt	attagtagaa	acaagggtat	ttttctttac	ctagctagg	5699

**THIS PAGE BLANK (USPTO)**



20. Juli 2000

**Claims**

1. A recombinant influenza virus for high-yield expression of incorporated  
5 foreign gene(s), which is genetically stable in the absence of any helper  
virus and which comprises at least one viral RNA segment being a  
bicistronic RNA molecule coding for two genes in tandem arrangement  
(tandem RNA segment), in said tandem RNA segment one of the standard  
viral genes being in covalent junction with a foreign, recombinant gene  
10 and said tandem RNA segment having an upstream splice donor and a  
downstream splice acceptor signal surrounding the proximal coding region.
2. The recombinant influenza virus of claim 1, wherein the tandem RNA  
segment contains one of the standard viral genes in distal mRNA position  
15 behind a foreign, recombinant gene in proximal position, or vice versa,  
both in antisense orientation with regard to the viral RNA as present within  
the virus.
3. The recombinant influenza virus of claim 1 or 2, wherein at least one  
20 of the regular viral RNA segments is replaced by a tandem RNA segment,  
preferably the replaced regular viral RNA segment is selected from the  
neuraminidase segment, hemagglutinin segment and NS segment.
4. The recombinant influenza virus of claims 1 to 3, wherein the splice  
25 donor and splice acceptor signals are selected from sequences as  
present in influenza WSN segment 7 and 8 or other partially effective  
splice reactin substrates.
5. The recombinant influenza virus of claim 4, wherein the splice donor  
30 and splice acceptor signals are selected from sequences as present in  
influenza WSN segment 7.

6. The recombinant influenza virus according to claims 1 to 5, wherein one or more of the regular viral RNA segments, differing from said at least one tandem RNA segment, comprises a vRNA encoding a foreign gene which may or may not be in covalent connection to one of the viral genes, and preferably one or more of the regular viral RNA segments has (have) been deleted and replaced by a tandem vRNA encoding in addition a foreign gene.
7. The recombinant influenza virus according to claims 1 to 6, in which the terminal viral RNA sequences of one or more of the regular segments and/or of the at least one tandem RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.
8. The recombinant influenza virus of claim 7, wherein the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides, and/or wherein the 13 nucleotide conserved influenza 5' terminal sequence has been modified by replacement of one or two nucleotides occurring in said sequence at positions 3 and 8 by other nucleotides.
9. The recombinant influenza virus of claim 8, wherein the replacements in the 3' terminal nucleotide sequence comprises the modifications G3A and C8U.
10. The recombinant influenza virus of claim 9, wherein the replacements in the 3' terminal nucleotide sequence comprises the modifications G3A, U5C and C8U, or G3C, U5C and C8G.

11.The recombinant influenza virus of claim 10, which comprises a 3' terminal nucleotide sequence of (5')-CCUGUUUCUACU-3'.

12.The recombinant influenza virus according to claims 7 to 12, wherein the 5' terminal nucleotide sequence comprises the modifications U3A and A8U resulting in a 5'-terminal sequence of 5'-AGAAGAAUCAAGG.

13. The recombinant influenza virus according to claims 1 to 12, which is a recombinant influenza A virus.

14.The recombinant influenza virus according to claims 1 to 13, in which the foreign gene(s) in the tandem RNA segment code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus.

15.The recombinant influenza virus according to claims 1 to 13, in which the foreign gene(s) in the tandem RNA segment code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of a B cell and/or T cell response.

16.The recombinant influenza virus according to claims 1 to 13, in which the foreign gene(s) in the tandem RNA segment is a nucleotide sequence causing viral attenuation.

17.The recombinant influenza virus of claim 16, wherein the foreign gene is coding for part of or for the entire viral neuraminidase gene in antisense orientation.

18.The recombinant influenza virus of claim 17, wherein the neraminidase gene in antisense orientation is attached to the hemagglutinin vRNA

segment, and optionally another gene or reporter gene is encoded in a second tandem vRNA, preferably in conjunction with NS2.

19.A method for the production of recombinant influenza viruses as defined in claims 1 to 18 comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in antisense or in sense tandem design,

(b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in the corresponding viral RNA segment, and

(c) thereafter selective vRNA inactivation through ribozyme cleavage.

20.A pharmaceutical composition comprising a recombinant influenza virus according to claims 1 to 18, preferably a recombinant influenza virus of claims 16 to 18.

21.Use of a recombinant influenza virus according to claims 1 to 18, preferably a recombinant influenza virus of claims 16 to 18, for preparing a medicament for vaccination purposes.

22.The use according to claim 21, wherein the medicament

(a) is suitable against influenza and/or against other infections;

(b) is present in form of inactivated preparations; and/or

(c) is present in form of live recombinant viruses.

23.Use of a recombinant influenza virus according to claims 1 to 18 for preparing agents for somatic gene therapy.

24. Use of a recombinant influenza virus according to claims 1 to 18 for preparing agents, for transfer and expression of foreign genes into cells infected by such viruses.

25. Use of a recombinant influenza virus according to claims 1 to 18 for preparing agents for transfer and expression of RNA molecules into cells infected by such viruses.

- 5 26. The use of claim 24, wherein the RNA molecules to be expressed are antisense sequences or double-strand sequences relative to the target cell cellular mRNA molecules, and/or the agent is suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

10

27. The use according to claims 23 to 26, wherein the agents are applicable in *ex vivo* and *in vivo* application schemes.

15

28. A method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus according to claims 1 to 19 as expression vector.

20

29. The method of claim 28, wherein the production is performed in cell culture cells or in fertilized chicken eggs.

30. A method for preventing and/or treating influenza which comprises administering an effective amount of a recombinant influenza virus according to claims 1 to 18, preferably of a recombinant influenza virus according to claims 16 to 18, to the mammal to be treated.

25

31. A method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus according to claims 1 to 18.

30

32. A method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method

comprises infecting the cells with a recombinant influenza virus according to claims 1 to 18.

33. Use of a recombinant influenza virus according to claims 1 to 18 for  
5 preparing agents for immunotherapy, preferably for autologous immunotherapy.

34. A method for an immunotherapy which comprises *ex vivo* infection of immune cells, preferably dendritic cells, with a recombinant influenza virus  
10 according to claims 1 to 18, and introduction of the transduced cells into the patient.

35. A method for the induction of antibodies which comprises utilizing a recombinant influenza virus according to claims 1 to 18 as an immunogen.

15

**Abstract**

The invention relates to recombinant influenza viruses for high-yield expression of incorporated foreign gene(s), which are genetically stable in the absence of any helper virus and which comprise at least one viral RNA segment being a tandem bicistronic RNA molecule coding for two genes in tandem, in said tandem bicistronic RNA molecule one of the standard viral genes being in covalent junction with a foreign, recombinant gene and having an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region.

The invention further provides a method for obtaining attenuated viruses which resist reassortment dependent progeny production in case of chance superinfections by wild-type influenza viruses; a method for the production of said recombinant influenza viruses; pharmaceutical compositions comprising said recombinant influenza viruses; and the use of said recombinant influenza viruses for preparing medicaments for vaccination purposes.





FIG. 1

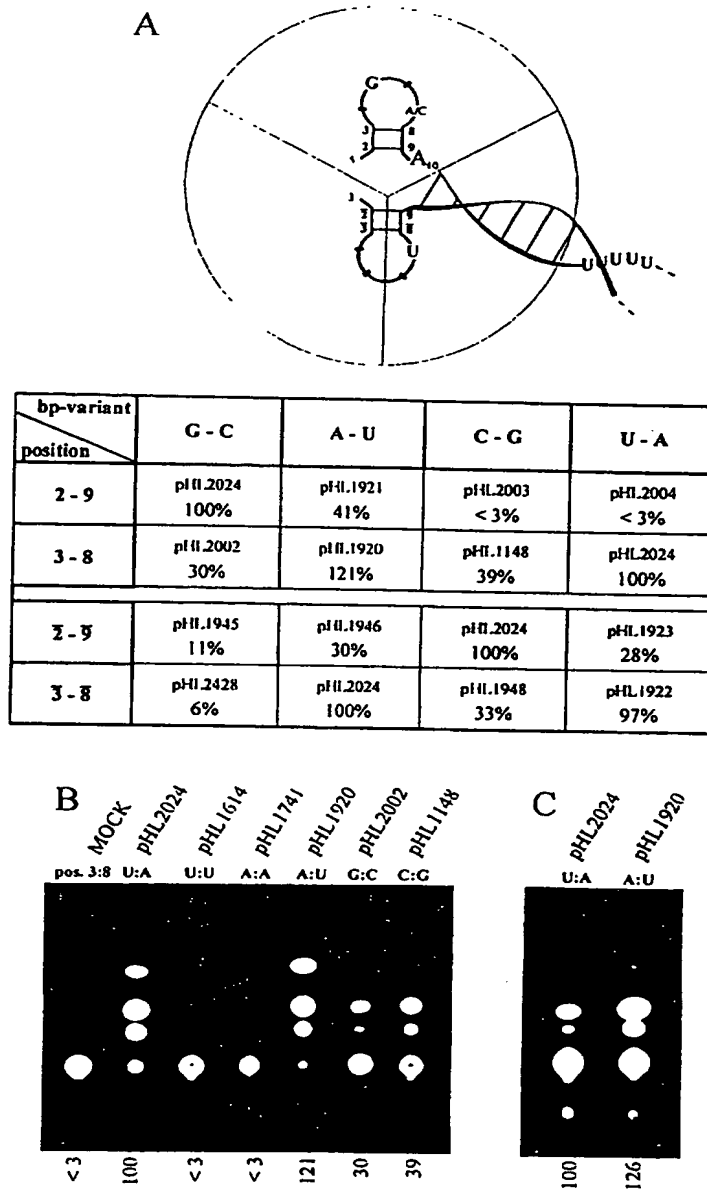


FIG. 2

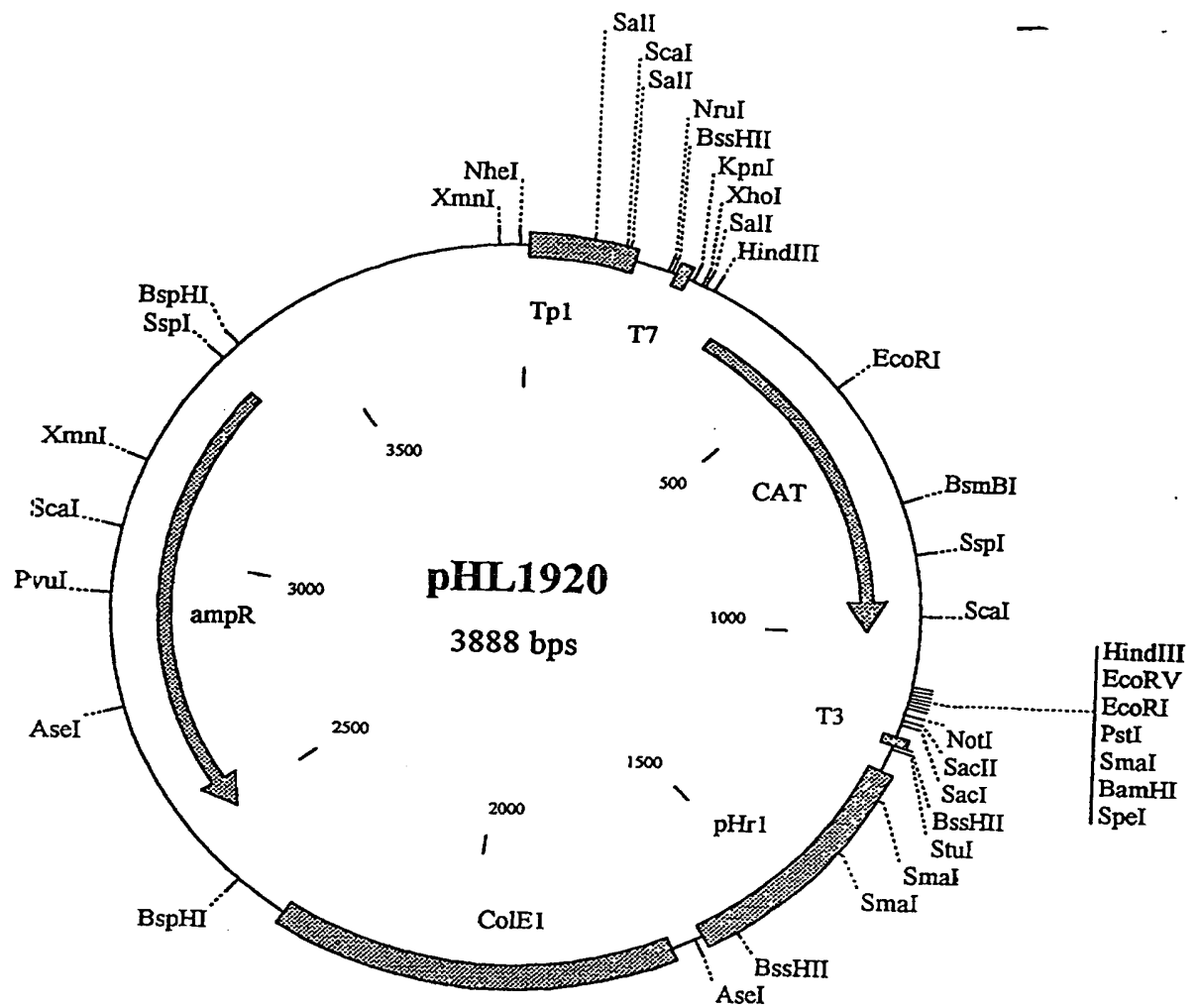
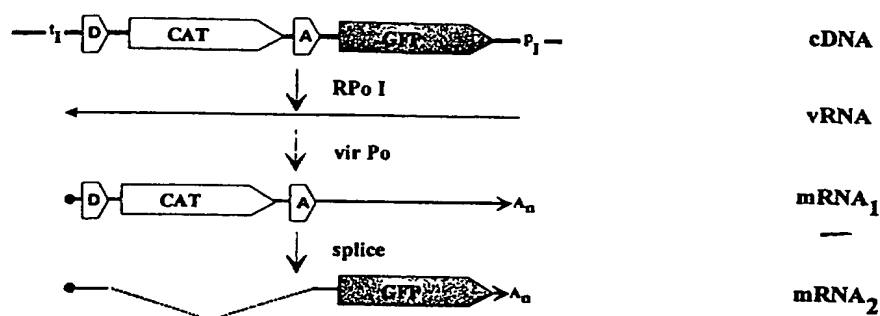
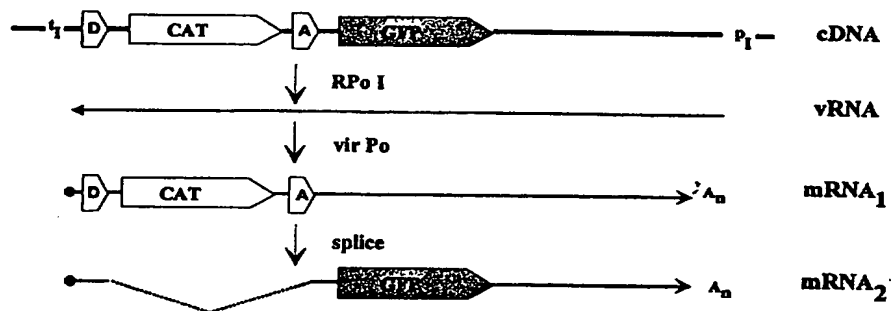


FIG. 3

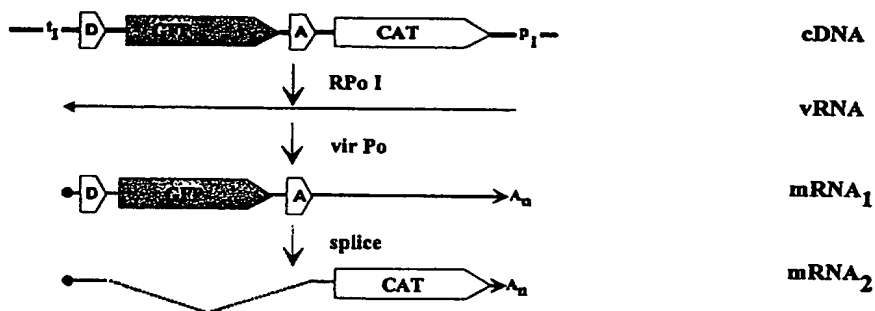
pHL3196



pHL 3235



pHL 3224



pHL3236

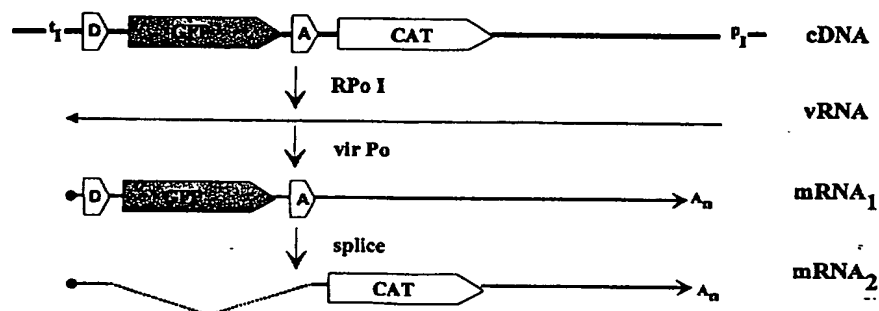
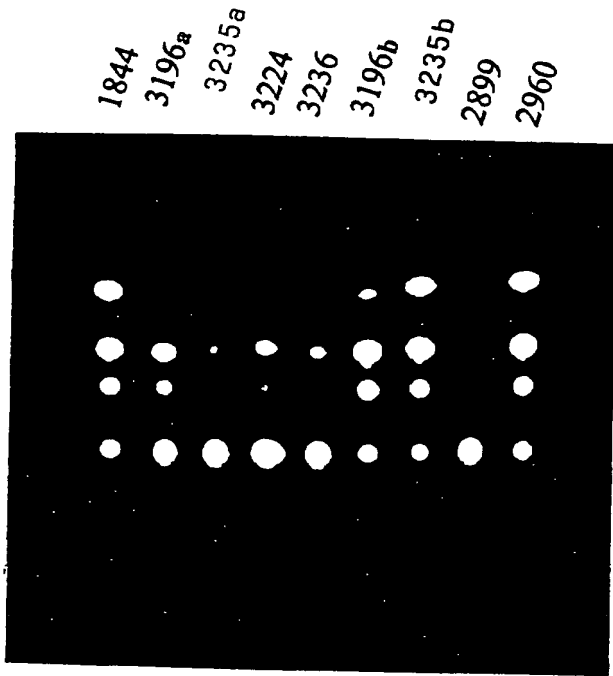
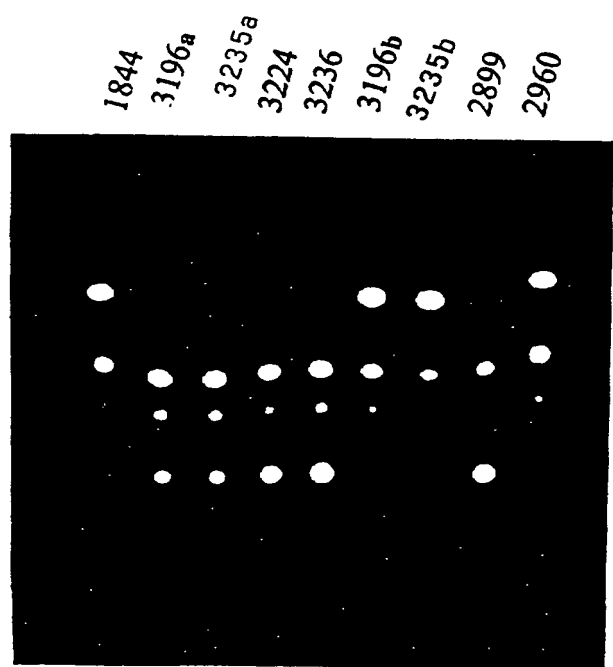


FIG. 4

A



B



**pHL3196**  
4500 bps

Key features and restriction sites:

- ampR**: Ampicillin resistance gene.
- CAT**: Catalase gene.
- Super-GFP(his)**: Super-GFP gene with histidine tag.
- ColE1**: Colony-forming efficiency (CFE) origin.
- Tp1 T7**: T7 promoter.
- Restriction sites**: SalI, HincII, ScaI, GsuI, Sall, HincII, EaeI, XmnI, BsaI, NspBII, PvuII, ApoI, GsuI, BstI, EaeI, DsaI, NcoI, NspBII, HincII, ApoI, GsuI, ApoI, EaeI, NdeI, BsrGI, Bst1107, BsgI, MunI, BsaI, BamHI, XhoII, Aval, HincII, EaeI, GsuI, EaeI, Aval, BssHII, AseI.

FIG. 6

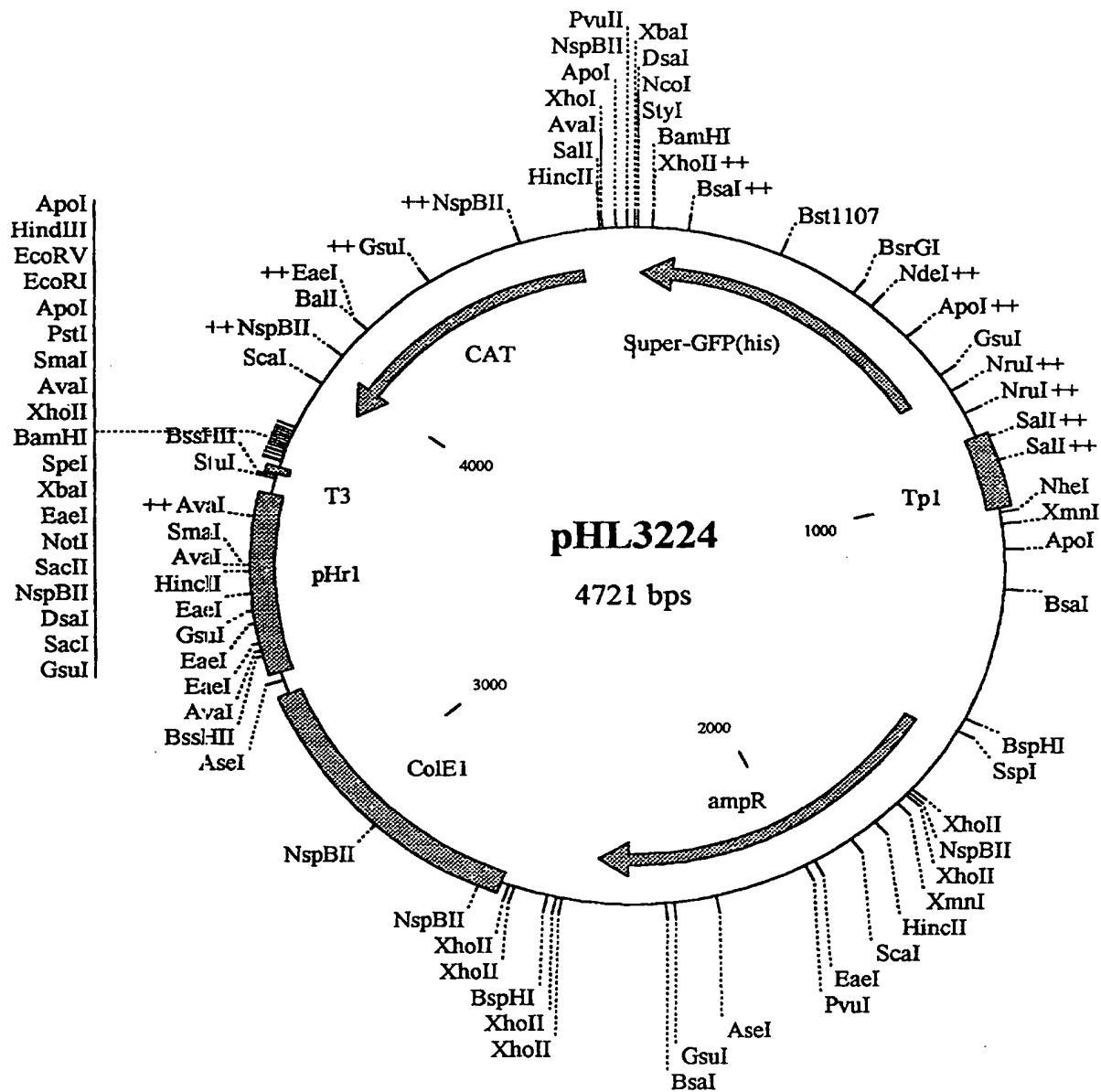


FIG. 7

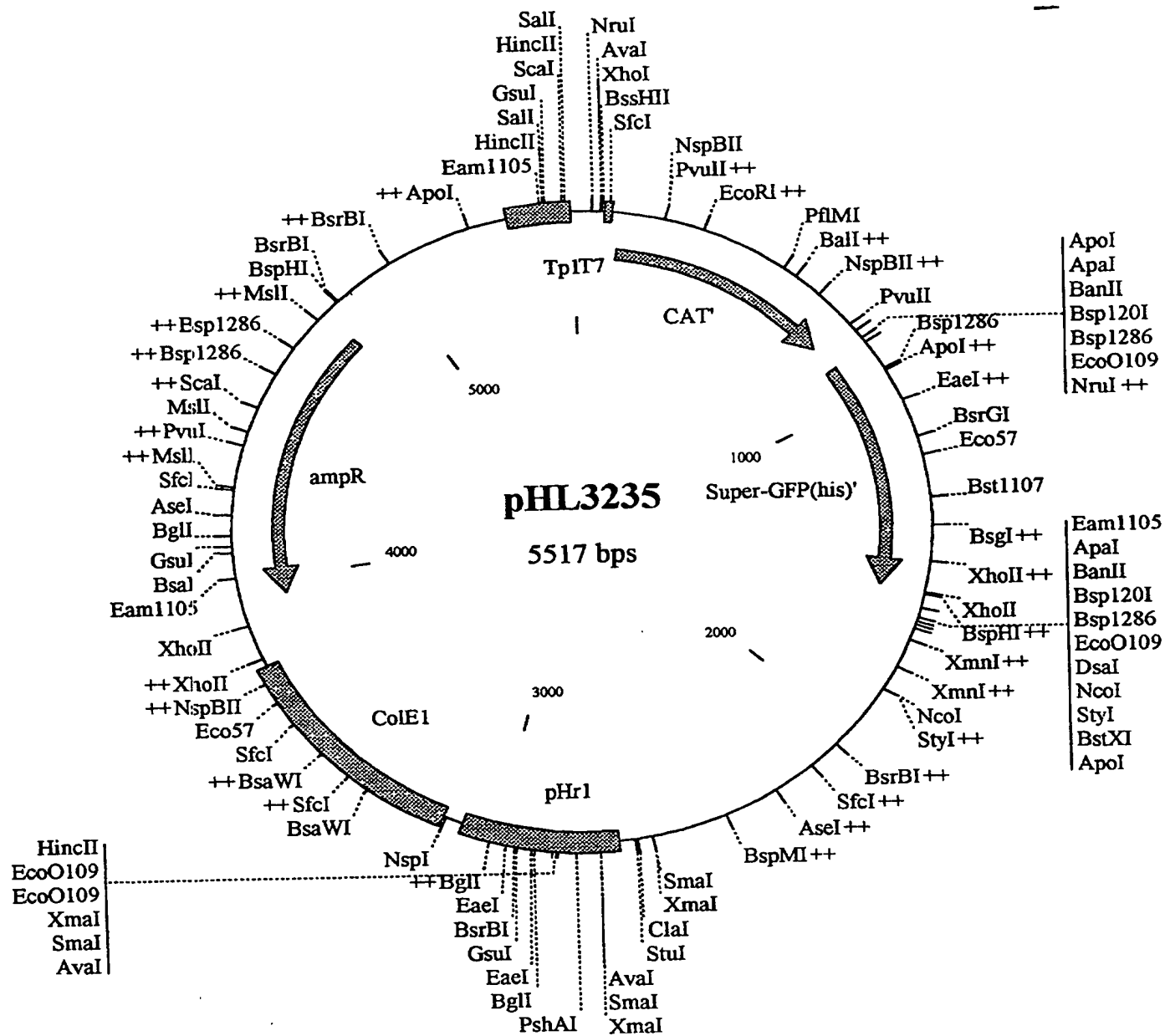
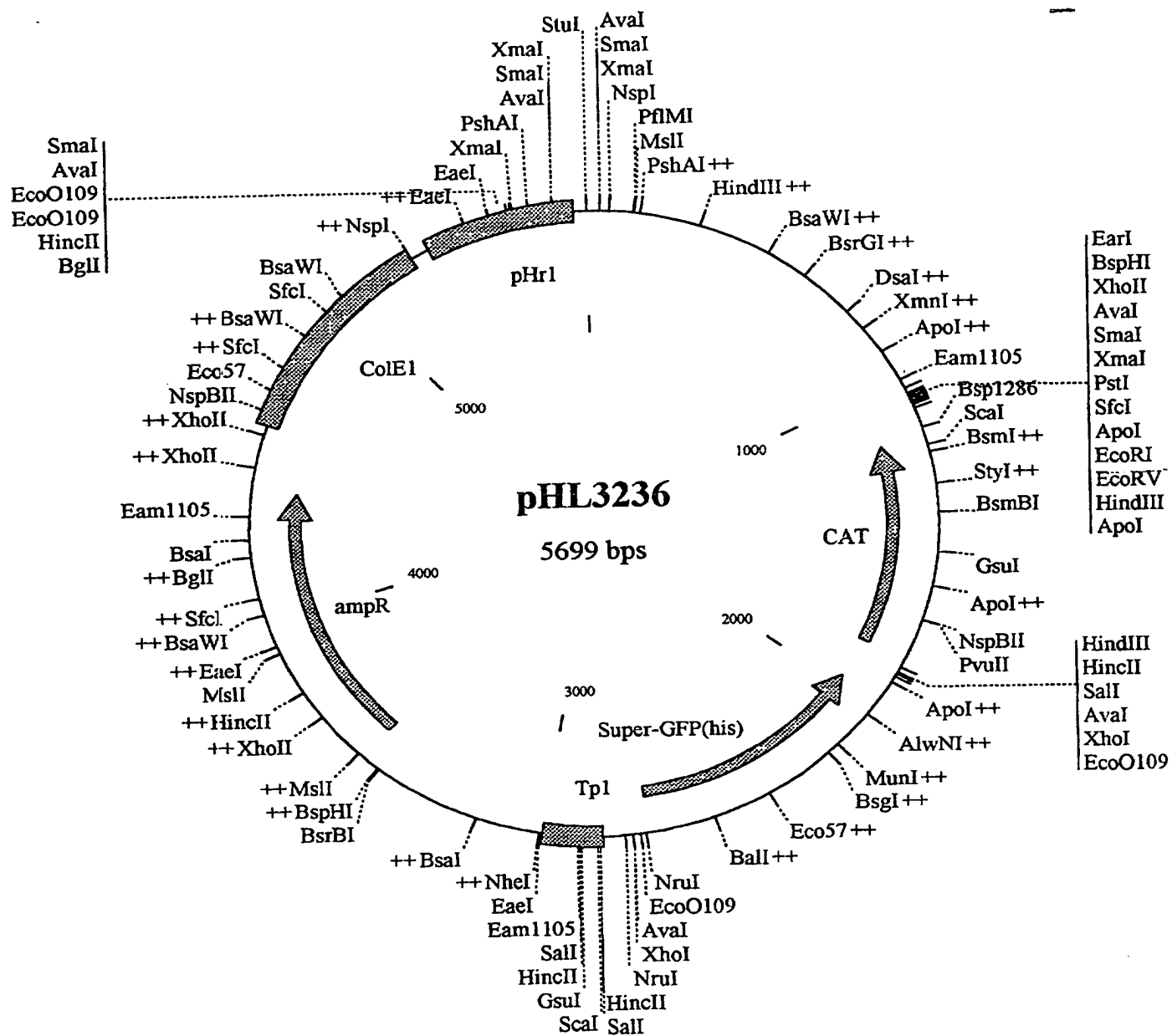


FIG. 8





**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**